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Experimental studies on *in vitro* germination of *Dioon edule* Lindl.

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Riassunto

È stata studiata la crescita *in vitro* di embrioni di *Dioon edule* isolati dal megagametofito ed i quali sono stati successivamente parzialmente o totalmente privati dei cotiledoni. Gli embrioni sono stati divisi in tre parti: (A) embrioni completi dei cotiledoni, (B) embrioni privi della parte fusa dei cotiledoni, (C) embrioni privi totalmente dei cotiledoni. Sono stati usati anche due diversi terreni di coltura solidi.

Sono stati studiati i rapporti tra megagametofito, embrione ed il ruolo dei cotiledoni. Sono stati osservati l'inverdimento e la crescita della parte libera dei cotiledoni e la progressiva essiccazione della parte fusa dei cotiledoni che conferma la loro funzione austoriale. È stato anche osservato che sia il megagametofito che la parte fusa dei cotiledoni non sono necessari per la germinazione; inoltre il megagametofito sembra non essere una fonte di fattori di crescita nello sviluppo iniziale dell'embrione, ma è una importante fonte di nutrimento che migra verso la nuova pianta.

Introduction

Although there are several studies concerning seed germination in gymnosperms (Ball 1954, Berlyn and Miksche 1965, Brown and Gifford 1958, Bulard 1952, Sacher 1956), few studies have been carried out on the Cycadales, and in particular on the interaction between the megagametophyte, the growing embryo, and the role of cotyledons during embryo growth. Chamberlain (1935) reported that during germination of *Dioon edule* Lindl., the

Key words: *Dioon edule*, cotyledon excision, embryo culture, megagametophyte, *in vitro* development.

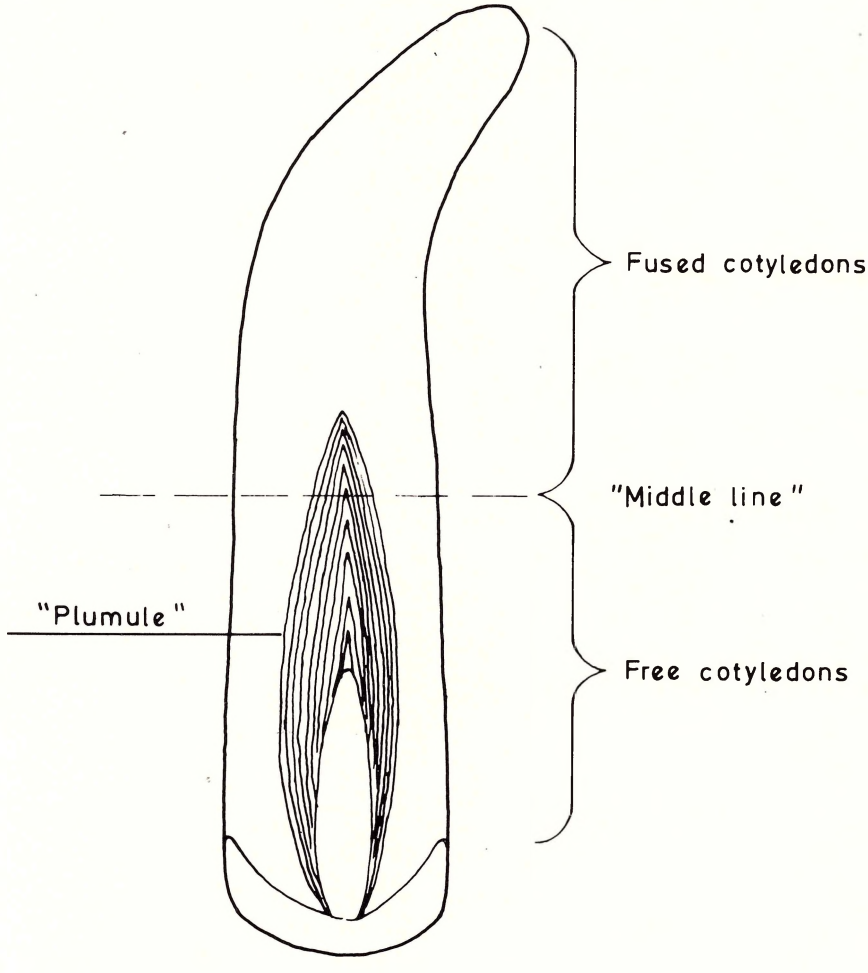

Figure 1 — Diagram of a longitudinal section of *Dioon edule* seed after integument removal.

cotyledonary tissue proximal to the shoot apex elongates and extends out of the megagametophyte, while the rest of cotyledons remain inside. The cotyledonary tissue inside the megagametophyte since then has been regarded as having an haustorial function.

Webb (1982) reported that the presence of the megagametophyte stimulated primary and secondary root elongation and se-

condary root production by *Zamia floridana* DC. (= *Z. integrifolia* Ait.) embryos. Moreover, Webb discussed the effects of partial and complete cotyledon excision on embryonic root growth in *Z. floridana.*

In order to examine relationship between megagametophyte, embryo, and the role of cotyledons, we conducted some experiments on the *in vitro* germination of *Dioon edule.* The embryo of *Dioon edule* consists of a root apex, a hypocotyl, a shoot apex, and two cotyledons which are free for 1/4 of the embryo proximal to the shoot apex and then are fused distally to form the remaining half of the embryo (fig. 1). In this paper, the growth of excised embryos and the effects of partial and complete cotyledon excision are examined.

Materials and methods

Seeds of *Dioon edule,* from Xalapa, Veracruz, Mexico, were supplied by Dr. Mario Vazques Torres of Universidad Veracruzana, Xalapa, Veracruz, Mexico.

Megagametophytes containing mature embryos (average length: 19.1 mm) were removed from the sclerotesta. Embryos were excised and sterilized in a commercial hypochlorite solution (15% v/v) for 20 minutes and rinsed with sterile water for three times. The material was divided into three lots: 1) whole embryos (A); 2) embryos with the fused portion of the cotyledons removed (B); 3) embryos with the total cotyledons removed (C).

Germination tests were carried out in: [1]- deionized water and [2]- 1/2 Murashige-Skoog medium, modified with sucrose (2% w/v). Sucrose and agar were added after adjusting the media to pH 5.7. Media were autoclaved at 115°C for 20 minutes. Sterile glass tubes of 30 mm in diameter filled with 50 ml of medium were used.

Whole embryos were placed horizontally or vertically with either the root tip or the fused cotyledons inserted in agarized glass tubes. Partial or complete cotyledon excised embryos were placed vertically with root tip or free cotyledons/shoot tip inserted in agarized glass tubes. At least 15 embryos were utilized for each experiment.

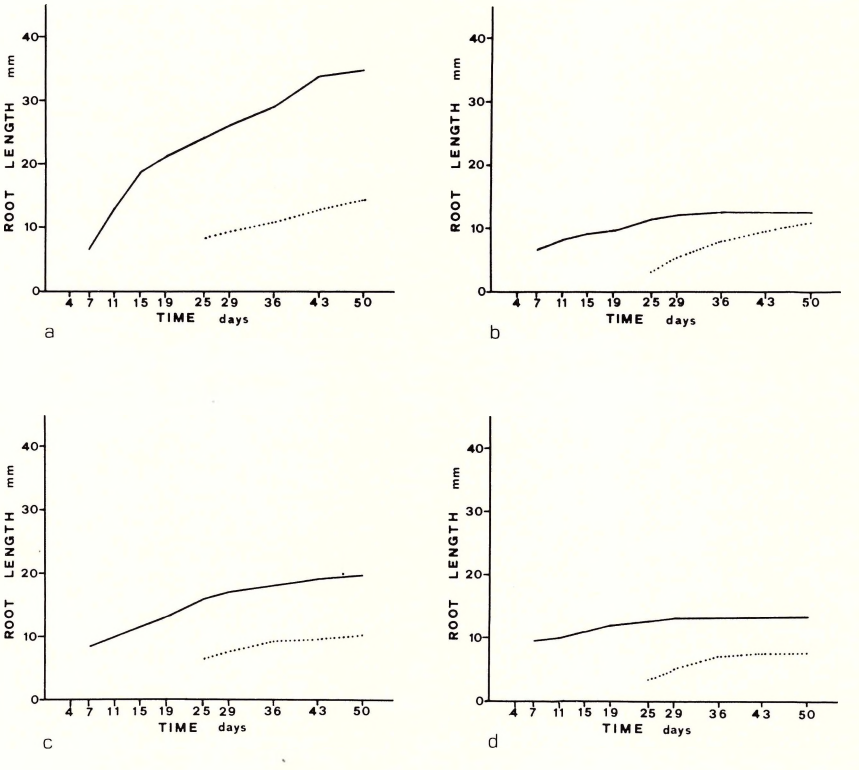

Figure 2 — *Dioon edule* embryo *in vitro*. Root elongation: embryo A with root tip in MS medium (a); embryo A with fused cotyledons in MS medium (b); embryo B with root tip in MS medium (c); embryo B with free cotyledons in MS medium (d). [light (...), dark (—)].

Cultures were grown at 30°C in dark or in light under continuous fluorescent light (Philips Daylight TLD58/55W) at 52 μE/m²/sec. Experiments lasted from the beginning of March 1985 to the end of May 1985.

Embryos were fixed in FAA (1:1:18, Formalin-Acetic acid-50% Ethanol v/v), dehydrated in toluene-ethanol series and embedded in Paraplast. 16 μm sections were stained in safranin and chlorazole black E.

Results

After the embryos were sterilized, a « line » became apparent between the free part and the fused part of cotyledons (plate I, fig. 1). This «line» became apparent, also, after immersion in water for few minutes. With light microscopy we did not observed any particular structure in this zone that would explain its presence and only observed that a number of canals run parallel to the longitudinal axis in the free part of cotyledons (plate I, fig. 2) which are not present at the level of this «line».

No growth differences were observed between the incubation media used in our experiments.

A) whole embryo: irrespective of the position of the embryos in the medium, the first evidence of growth consisted of a greening and increasing of the size of the free part of the cotyledons. The fused part of the cotyledons, on the contrary, maintained their size and after one week became dry and withered completely in one month. The free part of the cotyledons increased their length about 100%. In the case of embryos placed vertically with the fused cotyledons immersed in agarized glass tubes, the free part of cotyledons curved geotropically (plate II a). The cotyledons in the free part opened wide apart and the shoot apex became visible (plate II b). After this stage, the primary root became apparent. In the dark, these phenomena showed a delay of two weeks. At the end of the experiments, primary root elongation was greater in light than in dark (fig. 2 a-b). In some cases the cotyledons broke at the level of the «line» and callus regenerated at the surface of the free part of cotyledons (plate II c). The root elongation was much greater when root tip was immersed in the medium at the beginning of the experiment (fig. 2 a-b). The greening of the free part of cotyledons was greater in the embryos incubated at the light than in the dark. The leaf formation was observed only in one glass tube (plate III). However the leaf size was very reduced in comparison to the size of the first leaf produced *in vivo*.

B) Embryos with the fused portion of the cotyledons removed: embryos deprived of the fused part of cotyledons showed a pattern growth similar that whole embryos. The only difference consisted of a failure of a greater root elongation when the root tip of the embryo was immersed in the medium at the beginning

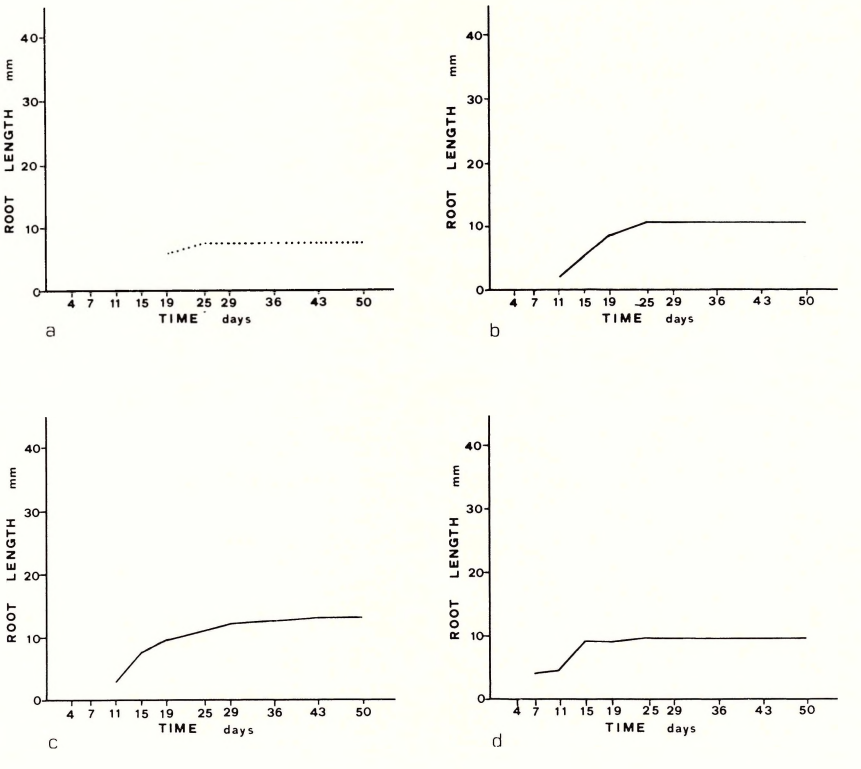

Figure 3 — *Dioon edule* embryo *in vitro*. Root elongation: embryo A in horizontal position in deionized medium (a); embryo A with fused cotyledons in deionized medium (b); embryo A in horizontal position in MS medium (c); embryo C with root tip in MS medium (d). [light (...), dark (—)].

of the experiment (fig. 2 c-d). We never observed callus regeneration.

C) Embryos with total cotyledons removed: root elongation (10% of the specimens) occurred only when the root tip was immersed in the medium (fig. 3 d).

Discussion

Our results demonstrate that the cotyledons of *Dioon edule* consist of two distinct parts that appear to have a different

role during the germination. While the free part of cotyledons appears to be important in primary root growth, the fused part of cotyledons probably has an haustorial function in the megagametophyte. However in our experiments this part of the cotyledons appears not to be required for germination and subseguent growth because whole embryos or embryos with the fused part of cotyledons removed show a similar pattern of development as those with intact cotyledons. The haustorial function may be suggested by the greater size of seedlings after germination *in vivo*. However, the free part of cotyledons appears very important, even if not indispensable, to promote root growth and development. In fact, we observed very little root formation in embryos with their cotyledons completely excised. In addition, it is apparent the elongation proximal unfused portion of the cotyledons is necessary to evict the root/shoot axis from the seed.

In *Dioon*, the megagametophyte does not play a crucial role during first stages of the germination. It appears not to be a required source of growth factors in promoting embryo development, but probably it is an important source of available nutrients that migrate into the new plant. In fact, the first leaf develops only after the primary root has completed its growth. This point of view is strengthened by the enormous size achieved by the primary root *in vivo*.

The autonomy of the embryo from the megagametophyte may be demonstrated by the normal seedlings development obtained when embryos were incubated in agarized deionized water.

Root growth and development is accelerated when embryos are incubated in light. Moreover, in all experiments root elongation appears to be stimulated by the light.

SUMMARY

Growth of excised *Dioon edule* embryos and the effects of partial and complete cotyledon excision were examined. The embryo were divided into three lots: (A) the whole embryos, (B) the fused portion of the cotyledons removed, (C) the embryos with the total cotyledons removed. Two different agarized media were used. We studied also the relationship between megagametophyte, embryo and the role of cotyledons. We observed greening and increasing of the size of the free part of

cotyledons and, on the contrary, the drying of fused part of cotyledons, and we could confirm the haustorial function of cotyledons. We also observed that megagametophyte and fused part of cotyledons were not required for germination. The megagametophyte appeared not to be a required source of growth factors in promoting embryo development, but it was important source of nutrients that migrate into the new plant.

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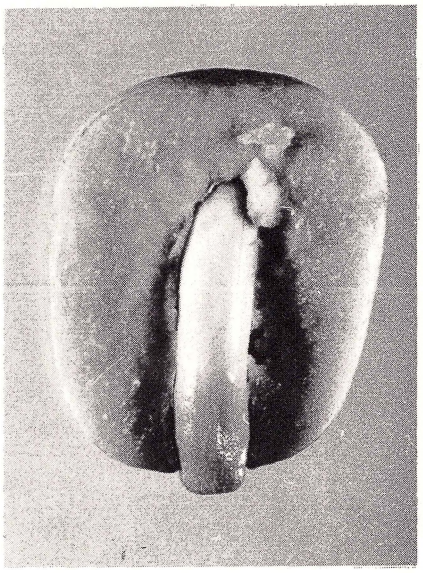
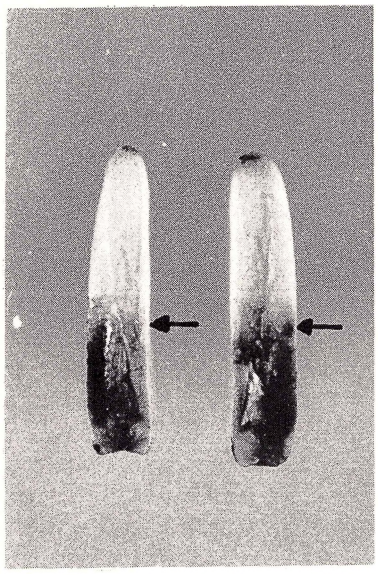
Plate I

Figure 1 — *Dioon edule* embryo *in vivo*. Arrow indicates the « line » between the free part and the fused part of cotyledons (× 2,5).

Figure 2 — Longitudinal section of *Dioon* embryo. Arrows indicate canals which run parallel to the longitudinal axis in the free part of cotyledons (× 15,6).





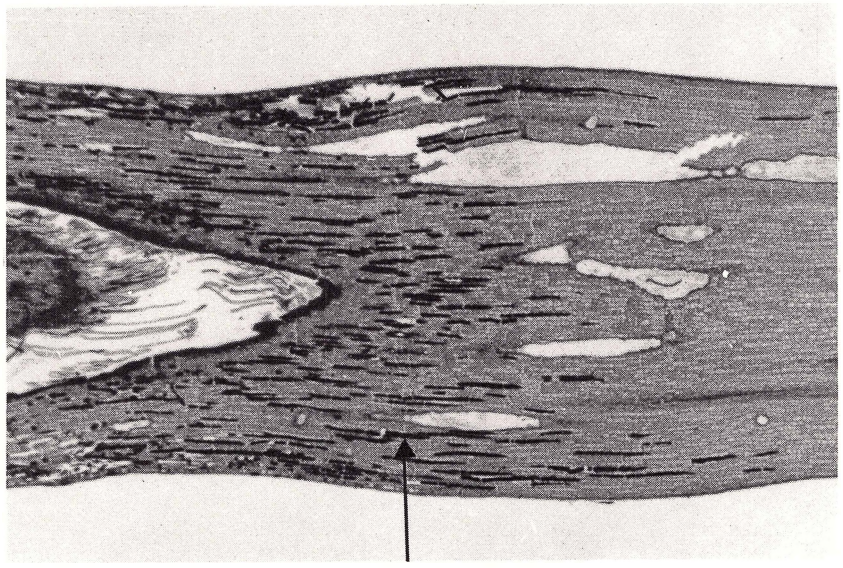


Plate II

Dioon edule embryo *in vitro* (a) with free cotyledons curved geotropically and primary root is curved and penetrated in the medium, (b) in the stage with the open free cotyledons and the shoot apex grows up, (c) callus regeneration (arrow) at the surface of the free part of cotyledons.

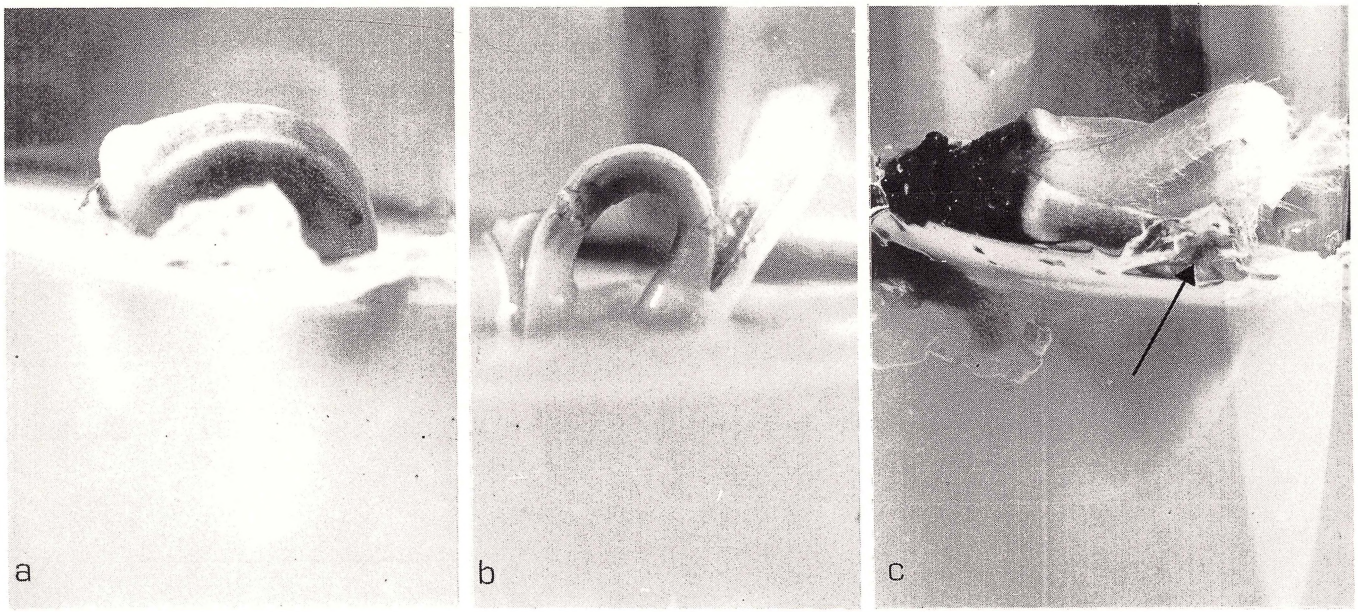

a
b
c

Plate III

First leaf in *Dioon* embryo grown *in vitro*. The leaf size is reduced in comparison to the size of the leaf *in vivo*.