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► **To cite this version:**

D. Cornu. Walnut somatic embryogenesis, physiological and histological aspects. *Annales des sciences forestières*, 1989, 46 (suppl.), pp.133s-135s. hal-02716371

HAL Id: hal-02716371

<https://hal.inrae.fr/hal-02716371v1>

Submitted on 1 Jun 2020

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Walnut somatic embryogenesis: physiological and histological aspects

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Introduction

Somatic embryogenesis in plant tissue cultures has been reported for many species. For mass cloning, embryogenesis could be better than organogenesis because it directly produces full plants instead of shoots that have to be rooted. Thus, one major problem in plantlet production could be bypassed. Moreover, embryogenesis will be a better way to apply other techniques, such as protoplast fusion or gene transfer.

Recently, the induction of somatic embryogenesis has included more and more woody tree species, important conifers and hardwoods (for a recent review see Tulecke, 1987). For the Juglandacea family, somatic embryos have been obtained with *Juglans regia*, *J. hindsii* and *Pterocarya* (Tulecke and McGranahan, 1985), *Carya illinoensis* (Merkle *et al.*, 1987), *Juglans nigra*, *J. major* and interspecific hybrids *J. nigra* x *J. regia* (Cornu, 1988). As for many other hardwoods, all results were obtained with immature seeds and a lot of abnormal structures or embryos were frequently observed.

The aims of this work are: 1) to determine the developmental and physiological stages able to give rise to somatic embryogenesis; and, 2) to control histologically the true nature of somatic embryogenesis at an early stage.

Materials and Methods

The nuts were provided by Mr. Germain (INRA-Arbiculture, Bordeaux) from a half-sib family collected on black walnut (NG 23), which naturally produces a high level of hybrid nuts. Nuts (40 at each time) were collected from the end of June to early October at 2-3 week intervals. They were not cold stored and culture began not more than 5 days after collection.

For all experiments, we used media described by Tulecke and McGranahan (1985). For the conditioning step, we also used the medium defined by Gupta and Durzan (1986) for spruce. Cotyledon sections were prepared and cultivated as described previously (Cornu, 1988).

For histological studies, suitable tissue samples were fixed in a formaldehyde-acetic acid-ethanol mixture, dehydrated, embedded in paraffin, sectioned and finally stained with safranin or fast green. For very soft callus tissue, small pieces were directly stained with safranin and observed after squash preparation.

Table I. Somatic embryogenesis from embryos or cotyledon tissue in relation to the development of nuts, after 4 weeks of culture on conditioning and induction media.

Sample date	Fruit development		Endosperm embryo + cotyledons	Reactivity in culture
	size (mm)	shell		
06/22	Ø 25 L 35	not lignified	liquid endosperm embryo ≤ 0.5 mm	no growth and death
07/15	Ø 35 L 45	hardened at tip	liquid endosperm embryo + cotyl ≈ 1–3 mm	soft callus necrosis
08/03	Ø 45 L 60	more than half lignified	gelified endosperm embryo ≈ 2 x 5 mm cotyl. ≈ 15–25 mm	callus growth 50% of culture ⇒ somatic embryos
09/01	Ø 45 L 60	totally lignified	no endosperm embryo ≈ 4 x 7 mm cotyl. filled shell	callus growth 1 callus gives somatic embryos
10/05	Ø 45 L 60	very hard	idem	callus growth but no organogenesis

Ø = diameter, L = length.

Results and Discussion

For all collection times, we have obtained somatic embryogenesis only at the beginning of August and in one case in September (Table I) and only on the Tulecke and McGranahan medium. This reactivity is connected with a particular stage of development of the nuts and occurs when the cotyledons show a high growth rate before they fill the locule. No cultures initiated from very early embryo stages, endosperms or mature embryos generated somatic embryos. This confirms the previous observations obtained on the reactivity of immature nuts of other walnut species (Tulecke and McGranahan, 1985; Cornu, 1988).

For *Juglans regia* the best stage for obtaining embryogenesis corresponds, in percentage of dry weight, to the highest content in protein and to the beginning of the decrease in soluble sugar and the increase in oil content (Labavitch and Polito, 1985). The interaction of the metabolic content of cotyledons and the culture medium could, in fact, play a major role in the induction or the development of induc-

ible cells in somatic embryogenesis. Consequently, we cannot be certain that our culture conditions were optimal for generating somatic embryos for more samples. In terms of growth regulators, auxins (normally not necessary for walnut micropropagation) are needed for the early conditioning medium (0.01 mg/l of indole butyric acid). This is in accordance with the results of Label and Cornu (1988), who found a particularly high concentration of indole acetic acid in the liquid endosperm at the corresponding stage of development.

During the first months of culture, the embryogenic lines gave compact white calluses similar to the original cotyledons. These calluses were embryogenic but in very limited areas. The adventive somatic embryos are frequently abnormal and do not complete their development to plantlets. Progressively, new calluses appear which are more irregular, soft, crumbly, moussey and which grow actively. In these calluses, we can distinguish different kinds of cells: 1) large elongated cells with a large vacuole; and, 2) small dense cells, like meristematic cells which gather to-

gether in micro- or macro-calluses. The structure of the callus depends directly upon the ratio of these 2 types of cells. The dense calluses show a very high capacity for embryogenesis. They generate large clusters of embryos at different stages of development (from the globular to the torpedo and even early cotyledonary form). At this late stage, embryos could be isolated, but to date only 20% complete their *in vitro* development with root and shoot growth. This heterogeneous callus development is similar to that described for conifers (Becwar *et al.*, 1988) except that elongated cells seem not to be active in the process of somatic embryogenesis (suspensor-like function). Finally, this kind of callus could be used to initiate agitated liquid cultures, which are easier to handle and more efficient for producing better and homogeneous somatic embryos.

Conclusion

We have obtained true somatic embryos in hybrid walnut. Our results indicate that there may be an optimal period of zygotic embryo development for the generation of somatic embryos. Not all zygotic embryos respond. Further investigations are needed to determine if an optimum physiological 'window' exists for initiating embryogenic cultures, or if other factors (particularly medium composition) limit the cell redetermination of more mature material. Analysis of liquid endosperm at all

stages of development of the zygotic embryo could be worthwhile in such investigations.

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