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Original article

Histological investigation of the multiplication step in secondary somatic embryogenesis of *Quercus robur* L.

Redouane Zegzouti, Marie-France Arnould and Jean-Michel Favre*

Unité Mixte de Recherche INRA-UHP Nancy, Interactions arbres/micro-organismes, Faculté des Sciences, BP 239, 54506 Vandœuvre-lès-Nancy Cedex, France

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Abstract – Standardized explants composed of hypocotyl and root-tip were prepared from embryonic structures obtained from one embryogenic line of *Quercus robur* L. maintained by regular transfer onto a solidified reference medium composed of the MS mineral solutions, glucose (0.12 M), casamino acid (0.1%), and NAA (10.74 µM). The regeneration capacity from these explants were tested on the reference medium and on 2 alternative media in which the NAA (10.74 µM) was omitted or substituted for a combination of IBA (9.80 µM)/BAP (8.90 µM). Within 30 days, 4 types of responses were observed including direct and indirect secondary embryogenesis. In the direct pathway, somatic embryos arose from 3–4 epidermal cells following two different modes, depending on whether or not the formation of a meristematic mass preceded the initiation of the embryogenic process. In the indirect pathway the embryos were formed from clumps of mitotically active cells included in callus developed within the cortical tissues. Depending on their histological origin, the embryos exhibited differences in their structural organization which could influence their potential for further maturation and conversion into viable plantlets. Explants prepared from small translucent embryonic structures were more embryogenic and expressed the direct pathway of secondary embryogenesis at higher frequency than explants prepared from more advanced embryonic structures. On the culture medium without growth regulator, direct secondary embryogenesis was the exclusive response whereas on the culture medium with growth regulators added both direct and indirect secondary embryogenesis occurred. NAA favoured the direct secondary embryogenesis, while conversely, the IBA/BAP combination stimulated the indirect secondary embryogenesis. The results are discussed in reference to the PEDC concept (pre-embryogenic determined cells).

secondary embryogenesis / growth regulators / histology / oak / PEDC

Résumé – Étude histologique de l'étape de multiplication dans le processus d'embryogenèse somatique secondaire chez *Quercus robur* L. Des explants standardisés composés d'un hypocotyle et du pole racinaire correspondant ont été préparés à partir de structures embryonnaires provenant d'une lignée embryogène de *Quercus robur* L. maintenue par repiquages réguliers sur un milieu de référence contenant les solutions minérales de MS, du glucose (0,12 M), de l'hydrolysat de caséine (0,1 %) et de l'ANA (10,74 µM). Les aptitudes à la régénération de ces explants ont été testées sur le milieu de référence et sur 2 milieux modifiés dans lesquels l'ANA a été supprimé ou remplacé par une combinaison d'AIB (9,80 µM) et de BAP (8,90 µM). Après 30 jours de culture 4 types de réponses différentes correspondant soit à une embryogenèse secondaire directe, soit à une embryogenèse secondaire indirecte ont été observés. Le processus d'embryogenèse directe débute à partir de 3–4 cellules épidermiques et se poursuit selon 2 voies différentes selon que la formation d'une masse tissulaire méristématique précède ou non la formation des structures embryonnaires. L'embryogenèse indirecte en revanche est initiée à partir de petits amas de cellules mitotiquement actives localisées dans les tissus corticaux sous-épidermiques. Selon leur origine histologique les embryons somatiques obtenus présentent des différences d'organisation morpho-anatomique qui peuvent influencer

* Correspondence and reprints
Tel. (33) 03 83 91 22 96; Fax. (33) 03 83 90 32 77; email: favre@scbiol.u-nancy.fr

leur maturation ultérieure et leur aptitude à être convertis en plantules viables. Les explants préparés à partir de structures embryonnaires petites et translucides sont plus embryogènes que ceux obtenus à partir de structures embryonnaires ayant atteint des stades de développement plus avancés. Sur le milieu de culture dépourvu de régulateurs de croissance l'embryogenèse secondaire directe est la seule réponse obtenue, alors qu'en présence de régulateurs de croissance les 2 voies d'embryogenèse peuvent être observées. L'ANA favorise l'embryogenèse secondaire directe et la combinaison d'AIB et de BAP stimule la voie indirecte. Ces résultats sont discutés en référence au concept de détermination pré-embryogène des cellules (PEDC).

embryogenèse secondaire / régulateurs de croissance / histologie / Chêne / PEDC

1. INTRODUCTION

Somatic embryogenesis involves control of 3 consecutive steps: (i) induction of embryogenic lines from sporophytic cells; (ii) maintenance and multiplication of embryogenic lines; (iii) maturation of somatic embryos and conversion into viable plantlets [47].

Many studies have been dedicated to problems of control and management of the initial establishment of embryogenic lines and the subsequent conversion step [41, 43, 46]. The multiplication step has been comparatively less investigated although it directly contributes to the final plant yield and influences the ability of the resulting embryos to germinate and develop into growing plantlets.

Two main problems have been reported concerning the multiplication step. The first one is the difficulty in obtaining stable and subculture-suitable lines that will produce embryos for long periods of time [43, 46]. The second is the lack of synchrony in embryo development and the risk of morphological abnormalities such as pluricotyledony, multiple apex formation, fused cotyledons and/or fasciation.

In angiosperm species, multiplication of embryogenic lines can be achieved either by regular subculturing of explants taken from compact or friable embryogenic calli [43], or by formation of new embryos from the previously developed somatic embryos themselves [3, 42, 46, 47]. This second case is referred to as secondary embryogenesis.

In *Quercus*, initiation of somatic embryogenesis has been described from a variety of sporophytic explants, namely stem segments, leaves and zygotic embryos. The multiplication of the embryogenic lines was first achieved from calli ageing on the same culture medium [12, 16] or via successive transfers onto fresh culture media with different growth regulator supplements [12, 14]. Embryogenic response from anthers and ovary tissues was also obtained using similar procedures [20].

Multiplication of embryogenic lines via secondary embryogenesis was most frequently accomplished using culture media containing the cytokinin BAP, with auxin NAA or IBA (*Q. suber* [4, 11, 12, 13]) or 2,4-D (*Q. robur* [5, 34]). More rarely BAP alone or in combination with GA_3 was used (*Q. petraea* [20], *Q. robur* [5, 34], *Q. acutissima* [39]). Zeatin alone or in combination with NAA was also used successfully in *Q. robur* [9].

Secondary embryogenesis on culture media without growth regulators has been reported for a number of species including *Q. rubra* [16], *Q. suber* [11, 14], *Q. acutissima* [22] and *Q. robur* [9, 34]. Fernández-Guijarro et al. [14] showed that on these growth regulator-free media, the secondary embryogenesis is influenced by macronutrient composition. Both low total nitrogen content and high reduced nitrogen concentration decreased the percentage of somatic embryos that expressed secondary embryogenesis.

None of these studies investigated the histological origin and structural organisation of the somatic embryos. However, researchers have noted that (i) within one embryogenic line the somatic embryos could occur from different histological origin, as observed for example in *Theobroma cacao* [1], (ii) the growth regulator composition of the culture medium influenced the histological origin of the somatic embryos (*Hevea brasiliensis* [30, 31], *Elaeis guineesis* [40]), and (iii) depending on their origin, somatic embryos exhibited different potentials for germination and further growth [30, 47].

In order to optimize the multiplication step in one *Q. robur* embryogenic line, we investigated the process of secondary embryogenesis from standardized explants, with special attention given to histological origin, early developmental stages and structural organisation of the resulting embryos.

2. MATERIALS AND METHODS

2.1. Plant material and explants preparation

The embryogenic line was established from one immature zygotic embryo at the beginning of cotyledonary

stage, which was excised from one acorn collected in July 1989 in the region of Heillecourt (Lorraine, NE France).

After a two month culture period, the excised zygotic embryo produced embryogenic tissue which was maintained by regular transfer onto fresh medium. This tissue continuously generated embryos which were used to prepare the explants. Two categories of embryonic structures were distinguished depending on their developmental stage. The first one consisted of 3–5 mm small

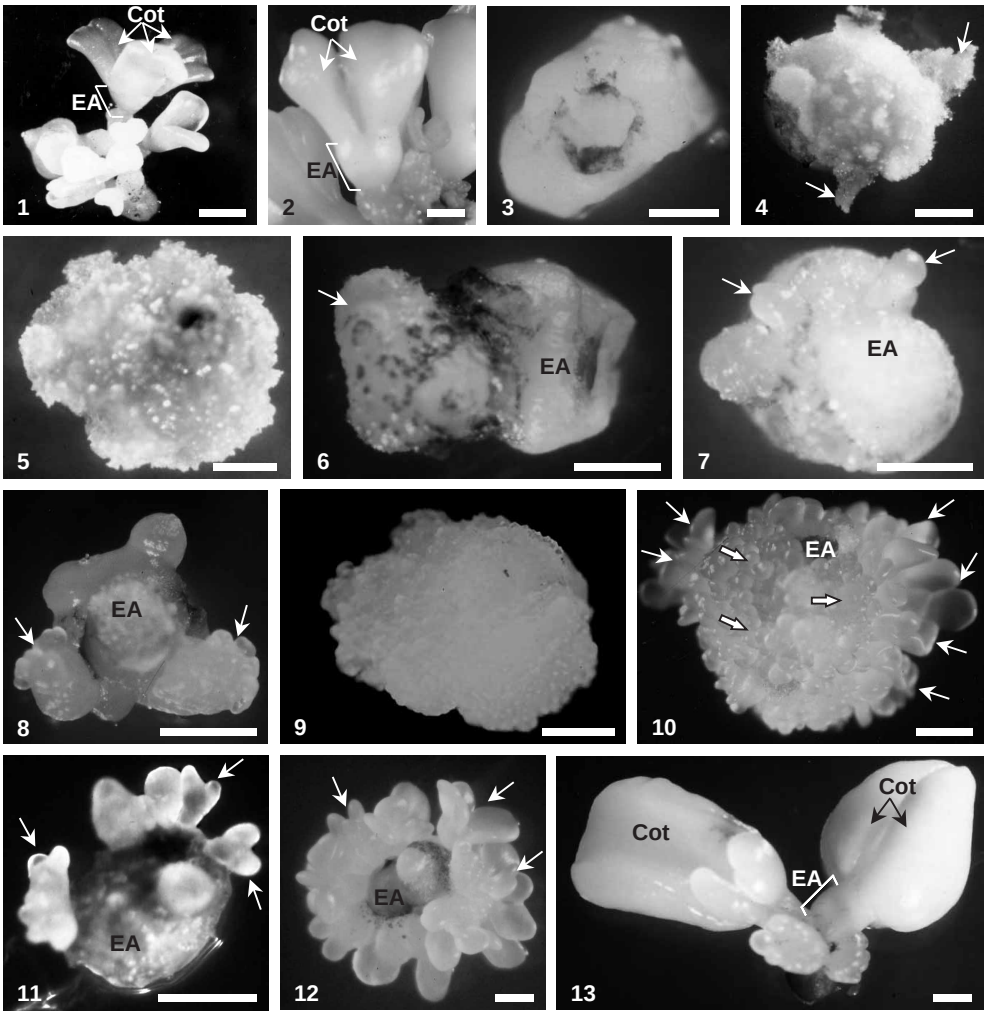

Figure 1.

1–2: Embryonic structures used for explant preparation (Bar = 1 mm). **1**. Small translucent structures (STE) with embryonic axis (EA) and several cotyledon pieces (Cot). **2**. Large white opaque embryos (LWE).

3–13: Explant responses (Bar = 1 mm). **3**. Response GI: swollen explant with intact epidermal surface. **4.** Response GII (early): explant with splitting epidermis and white callus extrusion (arrows). **5**. Response GII: advanced state showing complete disorganisation of initial explant and production of brown callus. **6.** Response GIII (early): callus proliferation (arrow) from embryonic axis (EA). **7**. Response GIII (advanced): globule formation on callus (arrows). **8**. Response GIII (final): emerging embryos showing initiation of cotyledons (arrows). **9**. Response GIV (early): swollen explant covered by small translucent globules. **10**. Response GIV (advanced): transformation of translucent globules (broad arrows) into small bipolar structures (fine arrows). **11, 12**. Response GIV (advanced): further development of translucent bipolar structures (heart stage) (STE) (arrows). **13**. Response GIV (final): white opaque embryos with large cotyledons (LWE).

translucent bipolar structures (noted STE; *figure 1-1*); the second of 5–7 mm white opaque structures with large cotyledons (noted LWE; *figure 1-2*). Standardized explants, composed of hypocotyl and root-tip (shoot-tip and cotyledons removed), were prepared from both these categories.

2.2. Culture media and conditions

The embryogenic line was propagated in Petri dishes (90 ×15 mm) on a solidified (Bacto-agar Sigma 0.8%) reference medium composed of MS full-strength macroelement and microelement solutions [32], glucose (0.12 M), casamino acid (0.1%), and NAA (10.74 µM) as growth regulator. The pH was adjusted to 5.5–5.6 before autoclaving at 120 °C for 20 minutes. Cultures were incubated at 25 °C in darkness and transferred onto fresh medium every 30 days.

Explants were cultured on the same reference medium and conditions as the embryogenic line. In addition two alternative media were tested. In the first one a combination of IBA (9.80 µM) / BAP (8.90 µM) was substituted for the NAA used in the reference medium, while in the second growth regulators were omitted.

2.3. Histological examinations

Explants were fixed using FAA [7] or the Randolph's CRAF solution [37]. Progressive dehydration in graded ethanol solutions (5 to 100%), clearing with xylene and embedding in paraffin were performed according to the traditional procedures.

Serial sections (5–7 µm) were stained either with Periodic acid-Schiff (PAS) [7] and Groat's hematoxylin [15] or with PAS and Naphthol Blue-Black [7].

3. RESULTS

3.1. Explants responses

Within 30 days, 4 types of responses were generally observed.

The first type (GI) was characterised by a slight swelling of explants without subsequent surface modification (*figure 1-3*). Culture for longer than 30 days did not result in further morphological changes and, after an additional 1–2 weeks, explants turned brown and died.

The second type (GII) corresponded to explants that showed internal tissue proliferation resulting in splitting of epidermis (*figure 1-4*), extrusion of brown callus and, finally, complete disorganisation of the initial explant (*figure 1-5*). When transferred onto fresh medium, these calli never expressed any organogenic activity and soon died.

In the third type of response (GIII), after initial swelling, explants produced hard, rough-surfaced and slow growing external callus (*figure 1-6*) from which a few globules were regenerated (*figure 1-7*). These globules secondarily developed into somatic embryos attached to the callus by a large basal connection (*figure 1-8*).

The fourth type of response (GIV) involved embryo production, but without preliminary callogenesis. The initial swelling step occurred as in response GIII, but the epidermis of the explants directly developed a number of small, smooth and translucent globules (*figures 1-9, 1-10*) that rapidly transformed into typical bipolar structures (*figures 1-10, 1-11, 1-12, 1-13*).

3.2. Histological investigation of secondary embryogenesis

Histological investigation was carried out for response types GIII and GIV which corresponded to two different secondary embryogenic pathways.

3.2.1. Indirect secondary embryogenesis

The initial evidence of indirect secondary embryogenesis consisted in cell divisions occurring within the cortical tissue (*figure 2-1*) that provoked the swelling of explants, the rupture of epidermis and the emergence of rough, hard and dry callus masses (*figure 2-2*). The inner region of these extruding callus masses was composed of radial alignments of vacuolated cells, while in the peripheral region, mitotically active cells (*figure 2-2*) formed growing globules which lacked epidermal layer (*figure 2-3*).

These globules differentiated bipolar structures with cotyledons and a stem-like axis lacking procambial strands, with large tissue connection to the supporting callus masses (*figure 2-4*).

No starch and/or protein storage was detected either within the callus masses, or in the cotyledons and hypocotyl-radicle axis.

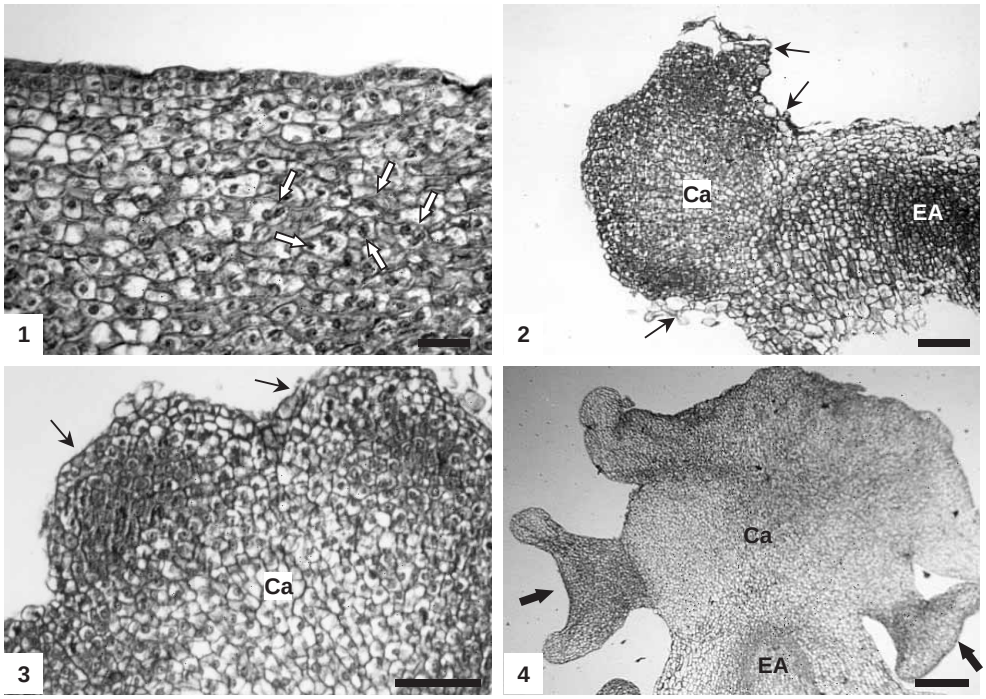

Figure 2. Histology of indirect secondary embryogenesis (GIII response) (PAS-naphthol blue-black). **1.** Transverse section of embryonic axis (EA) showing small clumps of densely stained cells in the innermost cortical tissue (arrows) (Bar = 50 µm). **2**. Section showing extrusion through the epidermis (arrows) of proliferating callus mass (Ca) with peripheral active and internal vacuolated cells (15 days culture) (Bar = 100 µm). **3**. Section of proliferating callus (Ca) showing emergence of globules without recognizable epidermal layer (arrows) (15 days culture) (Bar = 80 µm). **4**. Section showing embryonic structures (black arrows) formed from the proliferating callus mass (30 days culture) (Ca: callus; EA: explant axis) (Bar = 280 µm). Note the large tissue connection between callus and embryo.

3.2.2. Direct secondary embryogenesis

Direct secondary embryogenesis exclusively involved epidermal cells and occurred following two different modes.

In the first one, secondary embryogenesis began with synchronized periclinal cell divisions over large areas of the epidermis (*figure 3-1*). Cell divisions then progressively became asynchronous and lost periclinal orientation, thus producing compact, smooth-surfaced, meristematic masses clearly delimited by a protoderm (*figure 3-2*).

Within these growing meristematic masses, small individualized groups of about 10–50 cells delimited by thickened cell walls appeared (*figures 3-2, 3-3*) and developed into closely abutting proembryonic globules with well developed epidermis (*figures 3-4, 3-5*). These globules differentiated into embryos (*figure 3-6*).

In the second mode, each regenerated embryo resulted from the mitotic activity of a few number of epidermal cells (3–4). The first division plane was periclinally orientated (*figure 3-7*), the second one anticlinally orientated (*figure 3-8*) and then the following division planes occurred in any position, resulting in a small cluster of highly meristematic cells (*figure 3-9*). From these clusters of meristematic cells, nearly spherical globules with recognizable epidermis were formed (*figure 3-10*). These globules developed into typical embryos (*figure 3-11*) with convex meristematic shoot-tip and procambial strands connected to the cotyledon pieces in which starch accumulation could be observed at the end of the 30 days culture cycle. A root meristem was also observed (*figure 3-11*). The connection to the initial explant was small at the globule stage (*figure 3-10*) and completely disappeared at the end of the embryo development, so that the obtained somatic embryos could be easily removed.

3.3. Culture medium effects

After 30 days on the reference medium, almost all explants had produced either non-organogenic (GII), or embryogenic responses (GIII and GIV) (*figure 4-1*).

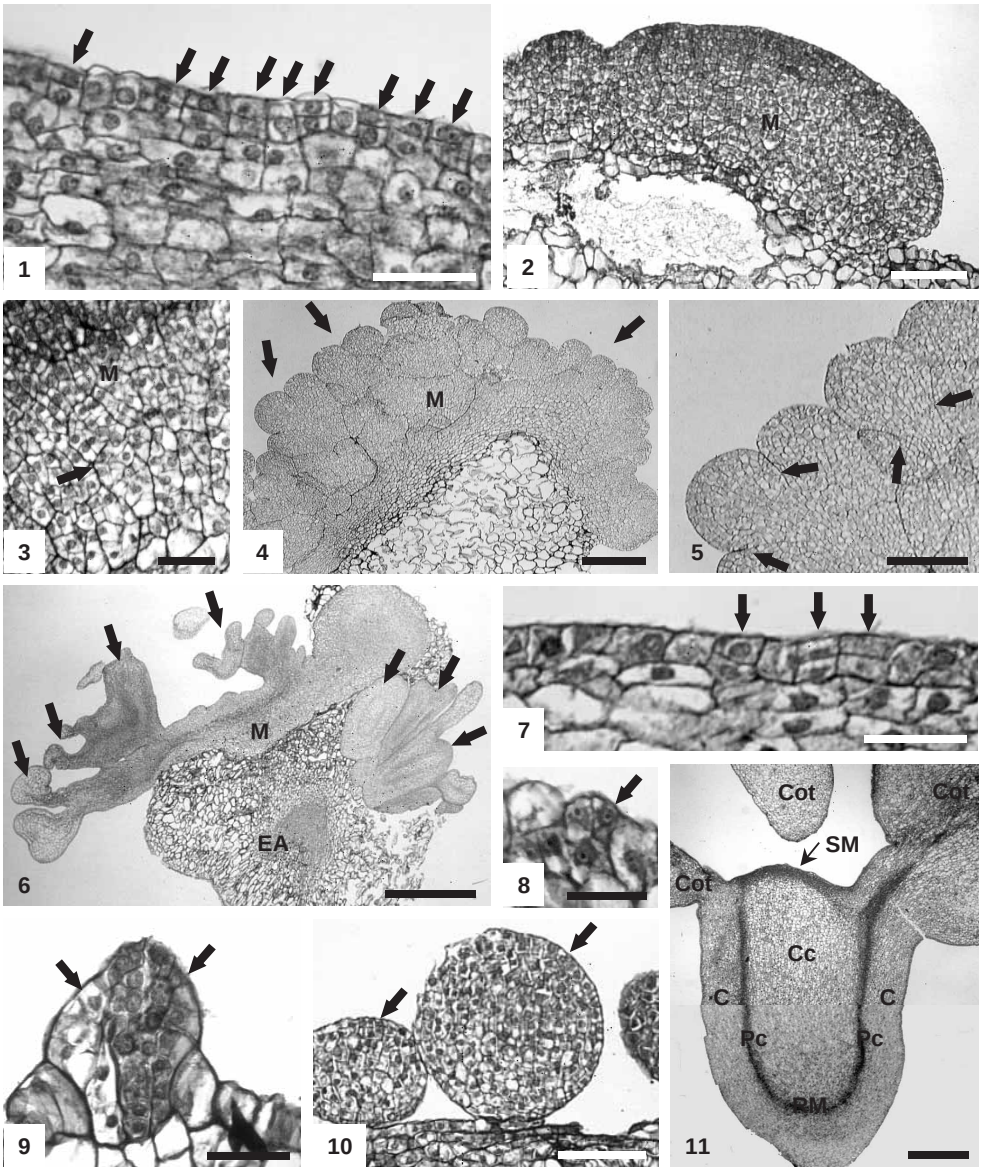

Figure 3. **Histology of direct secondary embryogenesis (response GIV).**

1–6: First mode (1-3 PAS-naphthol blue-black; 4-6 PAS-hematoxylin of Groat). **1**. Transverse section showing periclinal divisions (arrows) in explant epidermis (2 days culture) (Bar = 40 µm). **2**. Transverse section showing meristematic mass (M) formed from repetitive divisions in broad patches of epidermis (9 days culture) (Bar = 80 µm). **3**. Close view within the internal part of the meristematic mass (M) showing individualized groups of cells delimited by thickened cell walls (arrow) (9 days culture) (Bar = 40 µm). **4**. Differentiation of proembryonic globules (arrows) from the meristematic mass (M) (15 days culture) (bar = 280 µm). **5**. Close view showing separation of the proembryonic globules (arrows) (15 days culture) (Bar = 100 µm). **6**. Section showing the formation of bipolar structures (arrows) from the proembryonic globules (30 days culture) (Bar = 510 µm).

7–11: Second mode (PAS-naphthol blue-black). **7**. Transverse section showing periclinal cell divisions (arrows) in explant epidermis (2 days culture) (Bar = 40 µm). **8**. Detail of transverse section showing anticlinal division (arrow) of epidermal cell (6 days culture) (Bar = 30 µm). **9.** Transverse section showing a cluster of mitotically active cells (arrows) formed from a few number of epidermal cells (17 days culture, Bar = 40 µm). **10.** Proembryonic globules with well differentiated epidermis (arrows) (17 days culture) (Bar = 80 µm). **11**. Longitudinal section of somatic embryo after 30 days of culture. Cot: cotyledon; EA: embryonic axis; SM: shoot meristem; RM: root meristem; C: cortical tissue; Cc central cylinder; PS: procambial strands (Bar = 510 µm).

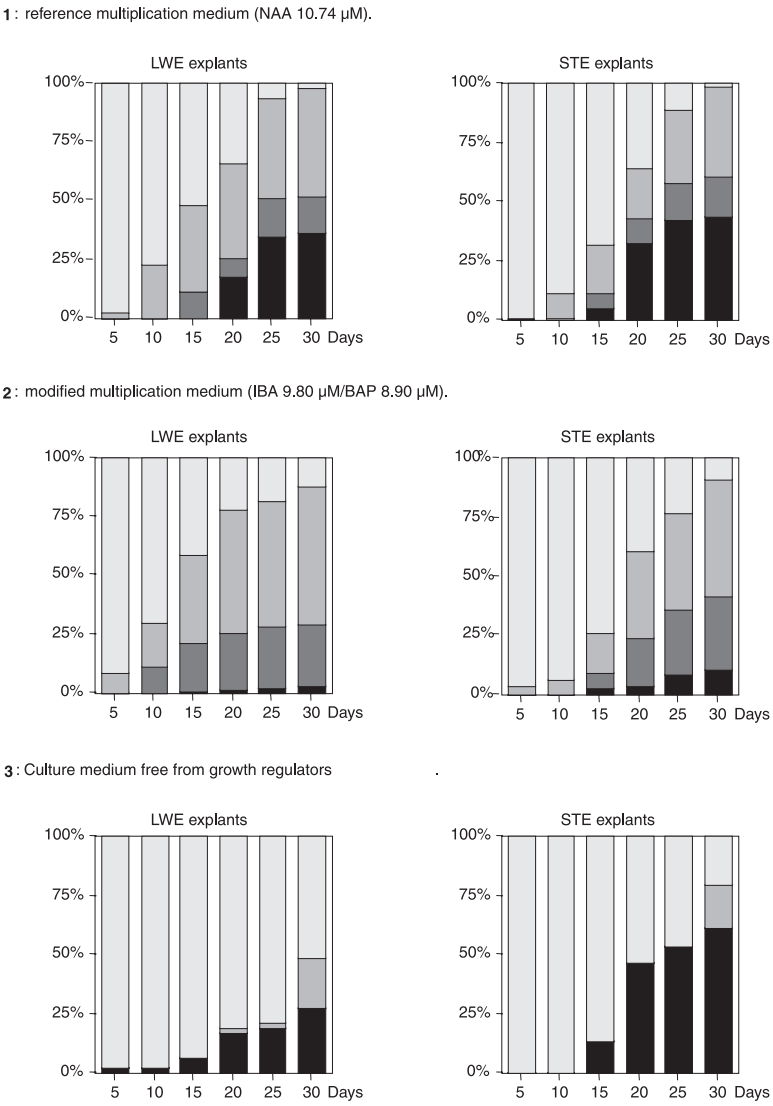

Figure 4. Percentage of GI, GII, GIII, GIV responses obtained from LWE and STE explants during one 30 days culture cycle (percentages calculated from 60 explants in 3 replicates).

Response type GI: swollen explants.

Response type GII: no organogenesis.

Response type GIII: indirect secondary embryogenesis.

Response type GIV: direct secondary embryogenesis.

Table I. Estimated number of embryos formed from 30 LWE or STE explants after 30 days on multiplication media containing NAA (10.74 µM) (reference medium), IBA (9.8 µM) / BAP (8.9 µM) or free from growth regulators.

	NAA		IBA/BAP		No growth regulators	
Explants	LWE	STE	LWE	STE	LWE	STE
Indirect secondary embryogenesis (GIII)	< 5	< 5	20–30	< 5	0	0
Direct secondary embryogenesis (GIV)	< 5	50–70	< 5	5–10	15–20	110–130

Response GII was first recorded by the end of the first week of culture, and finally reached 40–50% of explants. The embryogenic responses (GIII and GIV) occurred after 10–15 days and reached 50–60% of explants at the end of the culture cycle. The GIV responses were about 3 times more frequent than the GIII. The percentage of GIII+GIV responses was higher from the STE than from the LWE explant category. The preferential GIV response and the superiority of STE explants was also clearly visible when results were expressed in term of estimated number of embryos (*table I*).

Substitution in the culture medium of NAA (10.74 µM) by the IBA (9.80 µM) / BAP (8.90 µM) combination brought about a substantial decrease of the GIV response from both LWE and STE explants (*figure 4-2*). Conversely, the GIII response percentage increased approximately 2 fold. This increase did not balance out the reduction of the GIV response and the total percentage of embryogenic responses decreased from both LWE and STE explants. The best embryo yield was obtained from LWE explants (*table I*).

On the culture medium without growth regulator, responses observed from the STE and LWE explants strongly differed (*figure 4-3*).

Response from LWE explants was reduced compared to that obtained on the reference medium. After 30 days, 50% remained in stage GI, 20% turned brown (GII) and 25% produced somatic embryos. In contrast, the STE explants exhibited a high percentage of embryogenic responses, all of the GIV type. These results were confirmed in *table I*, which also showed better embryogenic potential from STE explants and exclusive GIV response type.

4. DISCUSSION

In the *Q. robur* embryogenic line studied in this paper, two pathways of secondary embryogenesis were described, depending on whether or not a callogenesis step occurs prior to initiation of the embryogenic process.

In the first one, the early signs of histological modification were observed within the cortical tissue of explants and could be interpreted as the first steps of dedifferentiation in parenchyma cells as mentioned in *Quercus suber* [10, 11] and other species [2, 31]. This resulted in the formation of calli composed of vacuolated cells and clumps of densely stained, mitotically active cells underneath the explant surface. These clumps of cells, which then produced embryos, could be identified as the irregularly segmented proembryonal complex formed after initial redetermination of cells in the non-zygotic embryogenic process of *Daucus carota* [17, 18] and *Trifolium repens* [47]. The resulting embryos were largely fixed to the calli, implying a probable multiple cell origin as previously found in *Daucus*, *Trifolium*, *Hevea* and *Coffea* [17, 18, 19, 31, 47]. They lacked provascular tissues and did not show any starch or protein body accumulation. This type of embryogenic process can be referred to as indirect secondary embryogenesis according to the definition given by Sharp et al. [41] and Wann [46].

The second pathway of secondary embryogenesis originated exclusively from epidermal cells which divided periclinally instead of following the normal anticlinal orientation. This change of pattern in the mitotic activity can be interpreted as the early expression of a new developmental sequence from epidermal cells which seem to be still embryogenically competent. The epidermal cells of the explants used in this study could thus be accepted as pre-embryogenic determined cells (PEDCs) as defined by Konar et al. [23], Sharp et al. [41], Maheswaran and Williams [27, 28] and Williams and Maheswaran [47]. The embryogenic process then proceeded classically via the formation of spherical globules delimited by epidermal layer, which further developed into typical embryos. These characteristics corresponded to the direct secondary somatic embryogenesis as described by Sharp et al. [41], Maheswaran and Williams [27, 28, 29] and Wann [46].

Depending on the number of epidermal PEDCs involved, two alternative modes could be recognised within this direct secondary embryogenic pathway. In the first one, repeated periclinal divisions affected large areas of the epidermal cell layer, thus amplifying the number of PEDCs. This resulted in the formation of compact meristematic masses which, in totality, transformed into closely abutting embryos possessing provascular system but no accumulated starch or protein bodies. Similar secondary embryogenesis was observed from *Q. suber* zygotic embryos, however with some below epidermis cells involved in the development of the compact meristematic masses [10]. By their histocytological characteristics and ability to be completely transformed into embryos, these meristematic masses, strongly evoked the proembryonal complex described in several herbaceous and woody species that formed single-cell origin somatic embryos from daughters of epidermal cells [17, 18, 19, 42, 47]. The second mode was characterised by the absence of epidermal PEDCs amplification. The embryogenic process arose from a small number of epidermal cells after a short step of periclinal mitotic activity. Remarkably, the obtained somatic embryos showed good structural organization, normal shoot meristem, provascular system and starch accumulation in the cotyledons as in zygotic embryos. Similar secondary embryogenesis has been described in many species including *Quercus suber* [10], *Juglans regia* [36], *Theobroma cacao* [1], *Feijoa sellowiana* [8], and herbaceous or monocotyledons such as *Daucus carota* [21], *Ranunculus sceleratus* [23], *Phoenix dactylifera* [44],

Panicum maximum [25], *Trifolium repens* [28] and *Panax ginseng* [6].

The balance between the expression of the direct versus indirect pathway of secondary embryogenesis firstly depended on the embryogenic competence of the epidermal cells. Indeed our results show that, whatever the growth regulators in the culture medium, explants prepared from small translucent embryonic structures (STE) were more embryogenic and gave direct secondary embryos at higher frequency than explants prepared from more advanced embryonic structures (LWE). This confirmed that the capacity for secondary embryogenesis is dependent on the non-differentiated state of the tissues which progressively disappears during growth and tissue specialisation as observed when zygotic embryos of *Quercus* [5, 13, 16, 22] and other species [29, 35, 43, 47] are used as initial explants.

Given the differentiation state of explant tissues, the obtained type of secondary embryogenesis was influenced by the composition of the culture medium, especially the growth regulators.

On culture medium free from growth regulators, the direct secondary embryogenesis was the exclusive response. When NAA was added results were similar to those obtained without growth regulators, showing high frequency of direct secondary embryogenesis. In the presence of IBA/BAP, the indirect secondary embryogenesis became the prevailing pathway, resulting in badly formed somatic embryos. NAA synthetic auxin alone therefore is compatible with the expression the direct pathway of secondary embryogenesis, whereas the IBA/BAP combination has adverse effects on the expression of PEDC capacity from epidermal cells.

It has been reported that the concentration of BAP or other cytokinins (5–10 µM) suppressed secondary embryogenesis or caused partial or complete inhibition of embryo development in cell suspension and tissue cultures [8, 24, 26, 33, 38, 45]. However, on cortical cells which are not directly in contact with the culture medium, the presence of IBA/BAP probably has a stimulating effect on their morphogenetic competence allowing subsequent callus induction and indirect secondary embryogenesis.

Using culture media formulated, especially in their growth regulator content, according to the histocytological organisation of standardised explants may therefore be a key point to gain a better control of the multiplication step of the somatic embryogenic process.

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