Histological analysis of indirect somatic embryogenesis in the Marsh clubmoss *Lycopodiella inundata* (L.) Holub (Pteridophytes)

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Abstract

An efficient in vitro plant regeneration method was developed for *Lycopodiella inundata* (L.) Holub, an endangered medicinal Lycopod (Pteridophytes). Vegetative apices were used as explant material. Nodular calluses were established after three cycles (13 weeks each) on a medium containing a few minerals and organic compounds and supplemented with 0.05 μM 3-indolebutyric acid (IBA) and 1.4 μM kinetin (Kin). Propagation was achieved every 13 weeks on this callus medium (CM). When nodular calluses were transferred on a medium supplemented with 2.5 μM IBA and 0.33 μM gibberellic acid (GA₃) designated as embryogenic medium (EM), organized structures appeared and developed into plantlets. Development phases were characterized by histological studies. Some phases of zygotic embryogenesis previously described for Lycopods were observed in *L. inundata*. Histological analyses established that an indirect somatic embryo was derived from a single embryogenic cell by following the zygotic developmental pathway. As this phenomenon has not previously been reported in Lycopods, a comparison between somatic and zygotic embryos is discussed based upon morphology and histology. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Callogenesis; Histology; Indirect somatic embryogenesis; *Lycopodiella inundata*; Pteridophytes

1. Introduction

Marsh clubmoss, *Lycopodiella inundata* (L.) Holub is commonly found in hygrophile and oligotrophic environments. It is an ancestral vascular plant that belongs to the Lycopodiales order which also includes *Huperzia selago* and *Lycopodium cernuum* [1]. Non-regulated collecting of these plants for industrial or pharmaceutical uses endangers their existence [1,2]. The wild population is a source of spores for condoms [3,4], and is traditionally used in practical medicine [5] and homeopathy [6]. New opportunities have been opened since alkaloids from Lycopodiales were tested for the alleviation of symptoms related to Alzheimer’s dementia [7] and as a drug against nerve agent toxicity [8].

In vitro propagation is currently initiated on ferns (Pteridophytes) from sporophyte fragments such as creeping stems. That process usually produces a typical nodular callus. Meristematic nodule propagation is also reported as an efficient method for in vitro plant regeneration of *Matteuccia struthiopteris* [9]. While ferns are often propagated by in vitro methods, no propagation via axenic spore germination or calluses derived from sporophyte has been described for Lycopods, including *L. inundata*.

According to its recent protected status as an endangered plant species, and because of the pharmaceutical importance of its alkaloids, it is important to micropropagate *L. inundata*. Axenic spore germination was not obtained, so fragments of the sporophyte were chosen as the explant. Vegetative

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**Abbreviations**: CM, callus medium; EM, embryogenic medium; GA₃, gibberellic acid; IBA, 3-indolebutyric acid; Kin, kinetin; MS, Murashige and Skoog medium; SE, somatic embryogenesis.

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apices of *L. inundata* were responsive. The protocol for propagation [10] was optimized. Organogenesis was studied using cytological techniques and concluded to result from the direct shooting from axillary meristems [10].

In this work, we report on the establishment of nodular callogenesis for propagation. The histological analysis of the initiation and development of somatic embryos is detailed and compared with zygotic embryogenesis in Lycopsids. Somatic embryogenesis (SE) among Plantae (Pteridophytes-Spermaphytes) is discussed.

### 2. Materials and methods

#### 2.1. Collection and sterilization of source material

*L. inundata* plants were harvested from a wild population in Villepail (Mayenne, France). Vegetative apices (3–5 mm) were excised from vegetative stems (Fig. 1A). Apices were sterilized as previously established [10].

#### 2.2. Callogenesis establishment

A total of five callogenesis media (CM-1 to CM-5) were used (Table 1). They contained full strength (CM-1, CM-2) or half-strength (CM-3 to CM-5) Murashige and Skoog medium (MS) [11] basal salts, 0.05 \( \mu \)M 3-indolebutyric acid (IBA) and 1.4 \( \mu \)M kinetin (Kin), and combinations of various additives (Table 1).

#### 2.3. Indirect somatic embryogenesis establishment

For inducing SE, calluses were transferred on the medium used for direct shooting of *L. inundata* from vegetative apices [10]. This medium was composed of: 100 g/l NH\(_4\)NO\(_3\), 100 g/l KNO\(_3\), 200 g/l KH\(_2\)PO\(_4\), 50 g/l CaCl\(_2\), 80 g/l MgSO\(_4\) and 1/4 MS microelements and was supplemented with 2.5 \( \mu \)M IBA and 0.33 \( \mu \)M gibberellic acid (GA\(_3\)). This medium is called embryogenesis medium (EM).

When each embryo developed into a plantlet characterized by two to three leaf cycles and a few roots, it was excised and transferred to a new EM to induce growth.

All media (CM and EM) were supplemented with 20 g/l sucrose and 7.5 g/l agar (Pronatec). The pH was adjusted to 5.4 and hormones were added to the media before autoclaving at 120°C for 20 min. All cultures were incubated at 20 ± 1°C, in light at 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) photosynthetic photon flux density and 14-h photoperiod.

#### 2.4. Histological analysis

Calluses were propagated by subculturing onto fresh callogenesis medium once every 13 weeks. To study starch evolution during callogenesis, callus fragments were excised from the same calluses each 9 days during 13 weeks.

Calluses were transferred to EM medium to study the SE. Callus samples were collected from the same calluses once a week during 13 weeks.

The samples were vacuum-infiltrated with FAE fixative (formaldehyde 37%; glacial acetic acid 100%; ethanol 50%, 6.5:3.5:100 ml) for 72 h at room temperature. Fixed tissue was dehydrated in a series of ethanol concentrations: 50, 70, 90, 100% (v/v) (30 min each bath). This material was vacuum-infiltrated with component A of JB-4 embedding plastic with catalyzer, and incubated at ambient temperature for 12 h. Components A and B were mixed together with catalyzer (30 min). The material was polymerized at 20°C in gelatin capsules for 50 min.

Thin sections (3 \( \mu \)m) were obtained using a Leica RM 2065 microtome. Sections were stained with PAS (periodic acid Schiff) or double stained with PAS and NBB (Napholt Blue Black). PAS specifically stained polysaccharides red [12], and NBB stained soluble and reserve proteins bluish-black [12,13]. Observations were made with a light microscope.

To assess the mitotic reactivity of callus cells during culture, the surface ratio of the nucleus and/or the nucleolus was achieved after staining. Measures were established on ten cells/section, for ten sections/sample distributed all along the sample.

### 3. Results

#### 3.1. Callogenesis propagation

#### 3.1.1. Callogenesis establishment

Most of the apices cultured either on mineral salts enriched media, or on organic additives enriched media (CM-1 to CM-4), turned yellow and
died after 3 weeks of culture (Table 1). When the medium was relatively poor in mineral salts and in organic components (CM-5), all apices survived (Table 1). After 13 weeks of culture, the initiation of callogenesis was observed in the meristematic zone of each surviving apex (Fig. 1B). The callus was obtained only after three cycles of 13 weeks of culture on CM-5. The CM-5 medium was chosen for callogenesis establishment and propagation, and is henceforth called the callogenesis medium (CM). Continuous propagation of callus could be obtained by subculturing on CM every 13 weeks. Initial callus (6–8 mm) was then subdivided into three to four small calluses (2 mm) which were separately cultured on CM for a new culture cycle.

Fig. 1. Establishment and morphological appearance of the nodular callus in *L. inundata* (L.) Holub. (A) Wild plant exhibiting vegetative apices (va) and sporangiferous apices (sa) in natural habitat, bar = 10 mm; (B) initiation of the callogenesis in the meristematic zone (mz) of the vegetative apex after one 13-week period on CM, bar = 500 μm; (C) nodular callus obtained after three cycles of subcultures on CM, bar = 500 μm.
Table 1
Effect of various additives and media composition on callus formation from vegetative apices

<table>
<thead>
<tr>
<th>Medium tested</th>
<th>Macro-elements</th>
<th>Micro-elements</th>
<th>Additives (mg/l)</th>
<th>% Of apices forming callus</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Myo-inositol</td>
<td>Thiamine</td>
</tr>
<tr>
<td>CM-1</td>
<td>MS</td>
<td>MS</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CM-2</td>
<td>MS</td>
<td>MS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CM-3</td>
<td>MS 1/2</td>
<td>MS 1/2</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>CM-4</td>
<td>MS 1/2</td>
<td>MS 1/2</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>CM-5</td>
<td>MS 1/2</td>
<td>MS 1/2</td>
<td>50</td>
<td></td>
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</tbody>
</table>

*Each medium was supplemented with 0.05 μM IBA and 1.4 μM kinetin. CM, callogenesis media; MS 1/2, Murashige and Skoog medium diluted twice. For each condition, 30 vegetative apices were cultured.

3.1.2. Morphological description

Since the 13th week of culture on CM (first period of the establishment), callogenesis was observed on the apical meristem and also on the axillary meristems situated at the basis of the four to five leaf cycles surrounding the apical meristem. During the second period, callogenesis extended along the explant only in the dorsal part. After three cycles of subculturing, calluses homogeneously consisted of nodules that were green and compact (Fig. 1C) and further designated as nodular callus. Each vegetative apex gives rise to one nodular callus (size 6–8 mm).

3.2. Histological analyses of callogenesis

Calluses were uniformly composed of non-vacuolated cells with prominent and central nuclei undergoing rapid cell division. Most of the cells exhibited various amounts of starch grains (Fig. 2A). Mitotic activity was intimately linked to the quantity of starch granules in the cytoplasm. During the S phase of the cell cycle, each cell contained ~20 starch granules, was 40 μm in diameter, and contained a nucleus of 20 μm with a surface ratio of 0.25 (Fig. 2B). The increasing size of the nucleus is due to the ploidy of *L. inundata* (2n = 4x = 156). The number of starch granules decreased to ten at the end of the telophase (Fig. 2C), then increased to ~40 during the interphase (Fig. 2D). During the interphase, the diameter of the nucleus was 12–13 μm and the surface ratio was 0.1. When calluses were cultured more than 13 weeks on the same CM, they needed more time to reinitiate mitosis, they multiplied very slowly and did not give rise to organized structures after the transfer to embryogenic medium (data not shown).

3.3. Somatic embryogenesis

3.3.1. Morphological description

After 2–3 weeks of culture on EM, domes emerged non-synchronously from the callus. Within 3–5 weeks, they evolved to a cylindrical shape and covered the callus surface (Fig. 3A). After 6–8 weeks, these cylindrical structures had an enlarged base and tapered top. They acquired a ‘leaf morphology’ (Fig. 3B). Within 8–13 weeks, each structure developed a plantlet consisting of numerous leaves and roots (Fig. 3C). These plantlets developed dichotomies similar to the wild *L. inundata* plants (Fig. 3D).

It was therefore established that organized structures could emerge from the callus surface, and undertake an autonomous development leading to a normal plantlet. Characteristic stages observed such as the cylinder form, leaves and finally the plantlet were remarkably similar to those previously described in literature on zygotic embryogenesis of Lycopodiales [14–16], and led us to initiate an histological study for confirmation.

3.3.2. Histological analysis of somatic embryos

Since the 1st week of culture on EM, cells at the callus surface exhibited specific features: high nucleoplasmic ratio, cytoplasm rich in proteins (stained in bluish-black with NBB), and prominent central nuclei with stainable and voluminous nucleoli (stained in bluish-black with NBB) (Fig. 4A). Cells were separated from each other by a thick wall that strongly stained with PAS. The first division was mainly asymmetrical and generated a small apical cell and a large basal cell (Fig. 4B). An outer thick wall surrounded the structure which did not show any cellular growth at this
stage. The apical cell underwent one anticlinal or oblique division to produce a three-celled structure of 40–50 μm in size (Fig. 4C). The next divisions were periclinal to the surface resulting in the formation of cylindrical multi-celled structures of ~70–80 μm (Fig. 4D). They were delimited by a thick polysaccharidic mucilage layer (2–3 μm in thickness) strongly stained by PAS, and presented one apical cell that was actively dividing. Within 4–5 weeks of culture, their growth led to a pyramidal shape (250 μm). Since the 6th week of culture, this structure appeared lengthened, became bulbous at its basis, and its top was tapered (Fig. 4E). A single-celled layer, poor in starch grains and devoid in proteins as no blue stain was detected, was located at the surface and surrounded the structure as a protoderm. From this stage, two parts were distinct: a basal bulbous zone consisting of cells rich in starch grains, and an apical tapered zone composed of vacuolated cells with neither starch grains nor soluble proteins located in the cytoplasm. Such a tapered zone is considered as an embryonary leaf. Between the 6th and 7th week of culture, the organized structures became more and more tapered, and showed a leaf structure (0.9–1 mm). They developed one lateral prolongation which helped the structure to emerge from the callus. Within the 7–8th week, an apical meristematic zone was noticed at the base of the embryonary leaf. This zone was constituted of small cells containing proteins. At the 8–10th week of culture, a first leaf cycle was produced on the structure (2–3 mm) (Fig. 4F). Then, 3 weeks later, the second leaf cycle appeared. Following this stage, one to two roots were formed and the plantlet (>3 mm) began to differentiate tracheids. These events occurred at a late stage.

Fig. 2. Histological analysis of the nodular callus during the propagation phase. Sections were double stained with PAS and NBB. (A) Nodular callus contains non-vacuolated cells exhibiting various amounts of starch grains (star), bar = 50 μm; (B) during S phase of cellular cycle, a prominent nucleus is observed in a central position and the number of starch grains decreased, bar = 10 μm; (C) during mitosis, and the end of the telophase, the number of starch grains is greatly reduced, bar = 10 μm; (D) during interphase, a smaller central nucleus (n) is surrounded by numerous starch granules, bar = 25 μm.
Each callus that was initially 3–7.5 mm in diameter, gives rise to ten to 23 plantlets which are isolated and transferred onto new EM to encourage their growth. More than 61% of plantlets were successfully acclimatized in the greenhouse. They remained green, developed new vegetative apices, roots and dichotomies. After 2 years in the greenhouse, all of them exhibited sporangiferous orthotropic apices, and appeared morphologically identical to the wild plants.

4. Discussion

The first signs of callogenesis were observed after 13 weeks of culture of apices on CM medium only in the meristematic zones. Histological analysis showed that the callus arose from the apical and axillary meristems. Axillary meristems also gave rise directly to shoots [10]. In this study, *L. inundata* vegetative apices were transferred onto EM after 11 weeks of culture on CM. Callus only formed at the dorsal part on the vegetative explant, opposite to the ventral part that forms roots.

The establishment of a homogenous nodular callus was possible only after three 13-week cycles of culture on CM. According to our histological studies and by comparison with another fern, *M. struthiopteris* (L.) Todaro [9], whole callus in *L. inundata* consisted of meristematic cells, and no parenchymatous cells were observed. No vascular
A connection was observed between the callus and organized structures.

Secondly, most of the phases described in *L. inundata* are morphologically similar to those observed in the zygotic pathway in some Lycopsids [14–16]. The cells designated as embryogenic cells

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**Fig. 4.** Histological study of the somatic embryogenesis: characterization of the main developmental stages. Sections were double stained with PAS and NBB. (A) Embryonic cells (large arrows) appear at the surface of the callus. They were easily distinguishable according to their small size and colour after double staining. Inner cells show large amount of starch (star). Bar = 20 μm. (B) First divisions occur (ink blot) inside the structure which does not grow. A thick wall (arrow heads) surrounds it. Bar = 10 μm. (C) Three-celled stage: an apical cell undergoes mitosis (ink blot) while a basal cell (bc) is more voluminous. Bar = 10 μm. (D) Cylinder stage: a thick mucilage (arrow heads) is located all around the embryo. The apical cells (asterisk) divide actively. Bar = 10 μm. (E) Pre-leaf stage: a bulbous zone (bz) is characterized by starch-filled cells. The tapered zone corresponds to the pre-leaf (pl) and contains no reserves. A protoderm (arrow heads) is differentiated and surrounds the embryo. Bar = 50 μm. (F) Young plantlet: an apical meristem (m) is present and is surrounded by the first leaf-cycle. The embryonary leaf (el) is still present. Bar = 200 μm.
in our calluses have been cytologically well-defined. A three-celled structure is observed after 1–2 weeks of culture on EM. An asymmetrical division was reported to produce a three-celled zygotic embryo in H. selago [14], and subsequent periclinal and anticlinal divisions gave rise to a cylindrical structure. A cylindrical stage was also observed in the multi-celled embryo of L. cernuum [15] like in L. inundata somatic embryos of 3–4 weeks. A cylindrical stage was also reported in SE in Asparagus officinalis L. (Liliaceae) [17].

A pyramidal stage has not been described in the literature on the zygotic pathway. We characterized a similar stage in L. inundata somatic embryos after 6 weeks of culture as Treub [15] described in L. cernuum, but considered it to be a pre-leaf step in L. inundata. An inclusion, designated as the foot, was noticed in L. inundata after 7–8 weeks of culture. In nature, the foot serves to hold the embryo to the prothalle [15], and such a step is also the only zygotic phase partially described in L. inundata in the literature [16].

The differentiation of an apical meristematic zone in the L. inundata plantlet was noticed, and it is assumed that in ferns, the apical meristem is a single pyramidal cell designated as the initial cell. Mitosis occurs on all its faces. The daughter-cells surround it and may undergo mitosis. In our Lycopodium, we did not observe a very distinguishable apical cell, but instead saw a group of small cells, strongly stained with NBB (for proteins).

The size (2–3 mm) of the Lycopodiales gametophytes in natural conditions [14–16] does not easily allow investigations, so zygotic embryogenesis is not well-defined for Lycopods. Information on zygotic embryogenesis was unavailable on Lycopodiales since the end of the 19th century. If we hypothesize that both the zygotic and somatic pathways are similar, as is assumed for Spermatophytes, L. inundata is a convenient material for such investigations.

As no other embryogenesis system exists in Pteridophytes, we focus on the other models available in Spermatophytes (Gymnosperms and Angiosperms) to highlight common or specific features of somatic embryogenesis among Plantae. Such a SE in a Lycopodiale member exhibits characteristics slightly different from those described in Spermatophytes. In Encephalartos cycadifolius, Encephalartos dyerianus, Encephalartos natalensis [18,19] and Ceratozomia mexicana [20], some primitive Gymnosperms, the first division of the embryogenic cell is asymmetrical, and produces an embryonary mass with a pseudo-suspensor. Polyembryonary is often observed and differs from our observations on Lycopodiella. However, they all need an extremely long time to cover all the steps for in vitro propagation.

In Angiosperms, a protoderm differentiates during the globular stage. In L. inundata, the globular stage is compared with the cylindrical structure where no histo-differentiation occurs. The protoderm appears later, simultaneously with the establishment of bipolarity (tubercle-leaf). Such polarity is quite different from the Angiosperm heart-embryo stage as L. inundata exhibits polarity with the starch-filled cells. Vascular tissues were present in earlier stages in Angiosperms than in L. inundata, where tracheids appear later in the plantlet (with the second leaf cycle).

Usually, it is stated that somatic embryos are derived either from a unique embryogenic cell or from a cluster of embryogenic cells well-characterized in cytology [12,21,22]. Cytological studies allow embryogenic cell characterization. When Spermatophyte embryos are of unicellular origin, the embryogenic cells are isolated from one another by an important modification of their cell walls, in particular gelification of the middle lamella, and undergo polarized division, thus forming globular proembryos [21,22]. In this work, we provide evidence for the unicellular origin of embryos. Moreover, embryogenic cells exhibit cytological features common to Lycopodiales (Pteridophytes) and some Spermatophytes.

In conclusion, this first report on SE in L. inundata (Lycopodiales, Pteridophytes), gives new insights to compare developmental biology especially in embryogenesis. From our knowledge, SE has not previously been described among Pteridophytes. Moreover, considering the callus propagation phase, and the Lycopod alkaloid functions for pharmaceutical purposes, sufficient masses of nodules could be produced for alkaloid extraction and characterization. Preliminary results on L. inundata alkaloids were encouraging (Atmane and Fliniaux, unpublished data).

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