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Original article

Histodifferentiation and ultrastructure of nodular cultures from seeds of *Vriesea friburgensis* Mez var. *paludosa* (L.B. Smith) L.B. Smith and leaf explants of *Vriesea reitzii* Leme & A. Costa (Bromeliaceae)

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ABSTRACT

Micropropagation *via* induction, multiplication and development of nodular cultures (NCs) is an efficient regeneration system for Bromeliaceae, a family of endangered monocot plants with ornamental value. Therefore, the present work aimed to induce NCs from seeds and leaf explants of *Vriesea* in order to characterize the morphological and histochemical aspects of induction and formation of these cultures. Seeds of *Vriesea friburgensis* var. *paludosa* were sterilized and inoculated into liquid culture media supplemented with different concentrations and combinations of growth regulators. Leaf explants of *Vriesea reitzii* were inoculated into medium supplemented with 4 μM α-naphthalene acetic acid (NAA) and 2 μM 6-benzylaminopurine (BAP). The addition of NAA (4 μM) in the culture medium used for seeds led to an induction rate of 72% in NCs. First, the embryo began to germinate, and afterwards, nodular structures started to form. While NCs formed from seeds is associated with root and shoot meristems, the formation of NCs from leaf explants involves the intercalary meristem. Meristematic cells generate an appropriate response in the induction medium, producing NCs by the proliferation of small cells with meristematic characteristics and large vacuolated cells. These results provide a better understanding of morphogenetic responses in bromeliads and, hence, the opportunity to develop optimized micropropagation protocols.

1. Introduction

In vitro regeneration, or morphogenesis, is the process by which new organs are formed from preexisting ones through either somatic embryogenesis or organogenesis [1]. The pattern of response of bromeliads to *in vitro* morphogenesis has different features in comparison to traditional regenerative systems. These regenerative systems were defined as nodule cultures (NCs) and have high

Abbreviations: BAP, 6-benzylaminopurine; 2-iP, N6 (2-isopentyl) adenine; CBB, Coomassie Brilliant Blue; CLSM, confocal laser scanning microscopy; MSB, MS basal medium; NAA, α-Naphthalene acetic acid; NCs, nodular cultures; PAS, Periodic Acid-Schiff; SEM, scanning electron microscopy; TDZ, N-phenyl-N′-1,2,3-thidiazol-5-ylurea; TB-O, Toluidine Blue O; TEM, Transmission electron microscopy.

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regenerative potential, culminating in the production of multiple adventitious shoots in suitable culture conditions [2]. NCs are globular meristematic cell clusters of a greenish-yellow color and with friable or slightly compact texture. NCs are regarded as an alternative morphogenetic pathway in plant tissue cultures [3,4], and as such, the development of nodular cultures has been described in several bromeliads, such as *Vriesea reitzii* [3,5,6], *Bilbergia zebrina* [4], and, more recently, *Ananas comosus* var. *comosus* [7]. Other systems similar to NCs in bromeliads have been described in other plants, including *Humulus lupulus* L. (Cannabaceae) [8,9], *Passiflora edulis* Sims (Passifloraceae) [10] and *Sclerocarya birrea* (Anacardiaceae) [11], but under different names, such as organogenic nodule cultures, organogenic nodules and nodular meristemoids.

One strategy for inducing NCs in bromeliads primarily uses leaf bases [3,6,7], although induction from nodal segments [4] and seeds has also been reported [12]. Moreover, similar structures were induced from floral segments [13,14]. Dal Vesco and Guerra [3] established an *in vitro* regeneration protocol based on the induction, multiplication and development of NCs of *V. reitzii.* That protocol was defined as an efficient strategy for micropropagation of bromeliads, which, although prized for their ornamental value, are, nonetheless, endangered. Likewise, Scherer et al. [7] used NCs of *A. comosus*, along with temporary immersion systems, to develop a protocol for large-scale micropropagation at low production costs.

These results point to the importance of studying the formation process, as well as the morphological and histological characterization, of NCs in order to deepen the understanding of morphogenetic response in bromeliads and enhance the chances of success in micropropagation procedures. Furthermore, the combined use of optical and electron microscopy can provide comprehensive insight into the morphology and internal structures of these cultures. Thus, the present work aimed to induce NCs in seeds of *Vriesea friburgensis* var. *paludosa* and leaf explants of *Vriesea reitzii*, followed by characterization of the morphological and histological aspects observed during the induction and formation of these regenerative systems.

2. Materials and methods

The basic growth medium used was composed of the saline formulation MS [15], supplemented with Morel vitamins [16] and sucrose ($30\,g\,L^{-1}$), hereinafter termed MS basal medium (MSB). The material was cultured on filter-paper bridges in test tubes containing 15 ml of liquid culture medium. Incubation conditions were described by Dal Vesco and Guerra [3].

2.1. Induction of NCs from seeds of Vriesea friburgensis var. paludosa

Seeds from mature fruits of *V. friburgensis* var. *paludosa* were collected from plants kept in the bromeliad collection at the Center for Agricultural Sciences (CCA), Universidade Federal de Santa Catarina (UFSC), and they were used as a source of explants for the induction assay (Fig. 1a).

Table 1
Treatments for induction of nodular cultures from *Vriesea friburgensis* var. *paludosa* seeds. BAP: 6-benzylaminopurine; 2-iP: N6 (2-isopentyl) adenine; NAA: α-naphthalene acetic acid.

Treatment	Growth regulators (μM)
T1	NAA (2)
T2	NAA (4)
T3	NAA (2) + BAP (2)
T4	NAA (2) + BAP (4)
T5	NAA (4) + BAP (4)
T6	NAA (4) + BAP (2)
T7	NAA (2) + 2-iP (8)
T8	NAA (4) + 2-iP (8)
T9	Without growth regulators

Decontamination was carried out according to the procedures described by Alves et al. [5]. The experimental design consisted of nine treatments, as described in Table 1. Each experimental unit consisted of 5 test tubes with 5 seeds each, arranged in a randomized complete block design with three replications. Data regarding the induction rate of NCs were collected after six weeks of culture. Original data were subjected to Analysis of Variance (ANOVA) and Tukey's mean separation test (5%). Based on the results of this experiment, the culture medium showing the highest induction rate of NCs was selected for subsequent morphological and histochemical analyses.

2.2. Induction of NCs from leaf explants of V. reitzii

Segments 0.4–0.6 cm in length containing the leaf basal region were excised from the 3rd to the 6th leaf of young shoots 2.0 ± 0.5 cm in height (Fig. 1b). The *in vitro* Collection of Bromeliads from the Laboratory of Plant Developmental Physiology and Genetics (LFDGV) at the CCA, UFSC, provided the young shoots that arose from successive subcultures in MSB medium. Leaf bases were inoculated into MSB liquid medium supplemented with NAA (4 μM) + BAP (2 μM), which was previously established by Dal Vesco and Guerra [3].

2.3. Morphological analysis of NCs

While the seeds and leaf bases were being cultured, data were collected and microphotographs were taken using a camera (Olympus® DP71) coupled to a stereoscope (Olympus® SZH-ILLB) in order to identify the events taking place during the induction and development of NCs.

2.4. Light microscopy

The samples were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions [17]. After dehydration, the samples were infiltrated with Historesin (Leica Historesin). Sections (5 μm) were obtained using a manual rotation microtome (Leica model RM 2135) with tungsten blades. The sections were stained with Toluidine Blue O (TB-O) 0.5%, pH 3.0, to identify acid polysaccharides [18]; Periodic Acid-Schiff (PAS) to identify neutral polysaccharides [19]; and Coomassie Brilliant Blue (CBB) 0.4% in Clarke's

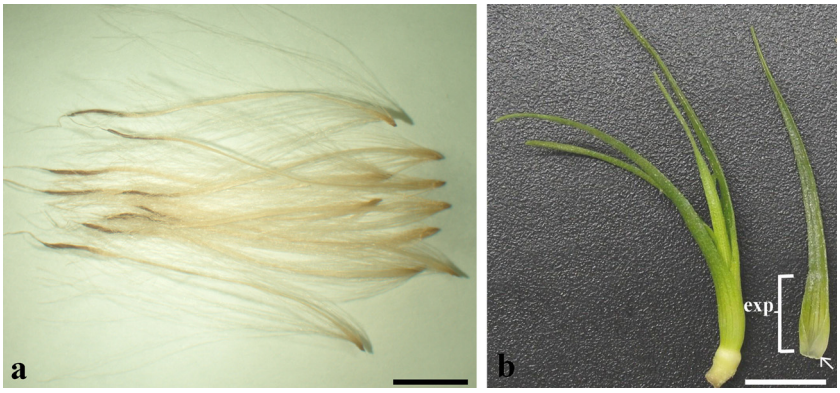

Fig. 1. Explants used for nodular cultures induction: (a) seeds of *V. friburgensis* var. *paludosa*; (b) leaf detached from the *in vitro* shoot, presenting intercalary meristem at the base (see arrow). Exp: leaf-base explant. Bar: 5 mm.

solution to identify proteins [19]. Some sections were double-stained with PAS + CBB [20]. Sections were analyzed with a camera (Olympus® DP 71) attached to a microscope (Olympus® BX-40).

2.5. Scanning electron microscopy (SEM)

Samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight. The material was post-fixed with 1% osmium tetroxide for 4 h [21]. The samples were dehydrated in an ethanol series, dried in a CO_2 Critical point dryer (EM-CPD-030, Leica), and then sputter-coated with gold prior to examination. The samples were examined under JSM 6390 LV scanning electron microscopy (JEOL Ltd.) at 20 kV.

2.6. Confocal laser scanning microscopy (CLSM)

The samples were analyzed in a confocal laser scanning microscope (Leica TCS SP-5) using an oil immersion HCX PL APO 63×/1.4–0.6 lambda objective lens. For nuclei detection, 0.5 mg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma–Aldrich, St. Louis, MO) was added to the samples during 40 min [22] and observed using a UV laser (peak wavelength of 405 nm excitation and spectrum emission of 510–566 nm). The LAS-AF Lite program (Leica) was used for final processing of the confocal images.

2.7. Transmission electron microscopy (TEM)

For observation in TEM, samples approximately 2 mm long were fixed in a solution containing 0.1 M sodium cacodylate buffer (pH 7.2), 4% formaldehyde and 2.5% glutaraldehyde for 12 h [17]. The material was post-fixed with 0.1 M sodium cacodylate buffer containing 1% osmium tetroxide for 4 h, dehydrated in an increasing series of acetone aqueous solutions, and then embedded in Spurr's resin [23]. Ultrathin sections (70 nm thick) were collected on grids and stained with aqueous uranyl acetate followed by lead citrate, according to Reynolds [24]. Sections were then examined under a JEM-1011 transmission electron microscope (JEOL Ltd.) at 80 kV.

3. Results

3.1. Induction of NCs from seeds of V. friburgensis var. paludosa

All treatments were effective in the induction of NCs from seeds of *V. friburgensis* var. *paludosa*. However, treatment T2 (NAA at 4 μM) showed a significantly higher ($p < 0.001$) induction rate (72%), whereas treatment T9 (devoid of growth regulators) showed the lowest (5.3%) induction rate after 6 weeks of culture (Fig. 2).

Germination started after three days of culture. Morphological changes were observed in seeds maintained 12 days in MSB medium supplemented with NAA (4 μM) and seeds maintained in culture medium without growth regulators (considered control) (Fig. 3a and b). This also happened in the histochemical analysis. The seeds in medium with NAA (4 μM) presented a more globular-shaped plantlet, featuring a protrusion in the root apical region with a greater amount of dense cells and thicker leaf primordia when compared to plantlets with normal development in medium without growth regulators (Fig. 3c and d). These cells revealed the presence of acidic

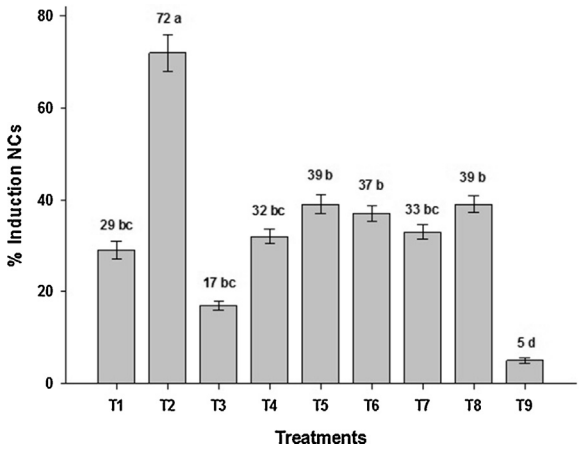

Fig. 2. Induction of nodular cultures from *V. friburgensis* var. *paludosa* seeds after 6 weeks of culture. Means followed by different letters are significantly different by Tukey's test (5%).

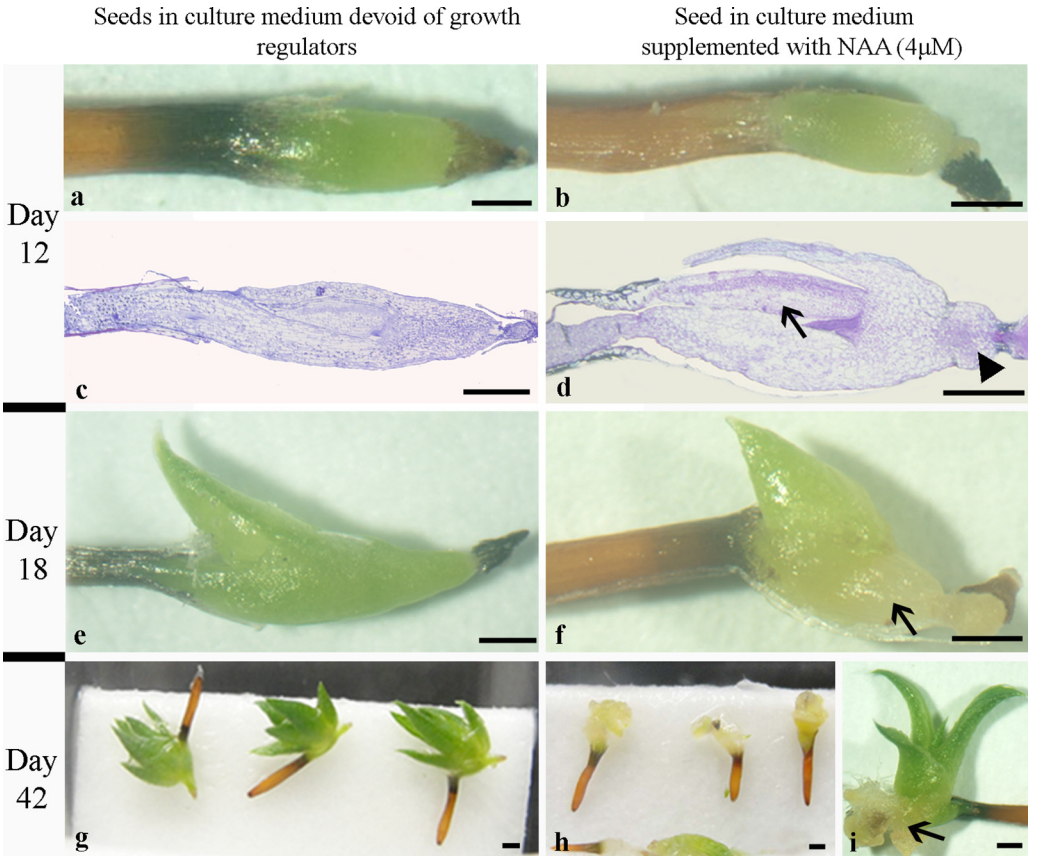

Fig. 3. Seeds of *V. friburgensis* var. *paludosa* on culture medium without growth regulators and on medium supplemented with NAA (4 μM): (a–b) 12 days of culture in medium; (c, d) longitudinal section stained with TB-O; (d) dense cells in the root apical meristem (arrowhead) and a big leaf primordium (arrow), (e, f) 18 days after inoculation into the medium; (e) plantlet with normal development; (f) formation of nodular cultures from the root apical meristem (arrow); (g–i) 42 days after culture in the medium; (g) plantlets with normal development; (h) Seeds forming nodular cultures; (i) plantlets with nodular cultures in the leaf base (arrow). Bar: 500 μm.

polysaccharides in the cytoplasm and on the cell wall by reaction to TB-O (Fig. 3c and d).

After 18 days of culture in medium devoid of growth regulators, the green cotyledon sheath emerged from the seed coat (Fig. 3e). However, seeds in medium with NAA (4 μM) showed cotyledon sheath with loss of greenness, turning to a greenish-yellow color. This event was characterized by the onset of inhibition of plantlet growth, followed by the formation of NCs (Fig. 3f). While seeds in control medium originated normal plantlets (Fig. 3g), NCs continued to proliferate, presenting a more intense yellow color after 42 days of culture (Fig. 3h). Some seeds in medium with NAA (4 μM) formed normal plantlets, but later showed induction of NCs at the base of the leaves (Fig. 3i).

Histochemical analysis on the 18th day of culture showed normal seedling development on medium without growth regulators (Fig. 4a). On culture medium with NAA (4 μM), these tissues showed loss of morphological characteristics caused by disorganized cell proliferation (Fig. 4b). Based on PAS reaction, neutral polysaccharides were found primarily as cell wall constituents, indicating the presence of cellulose and/or pectin (Fig. 4b). Cells that were double-stained with PAS + CBB showed blue-colored prominent nuclei and red-colored starch grains in the cytoplasm. Different cells were noted, some of which showed meristematic characteristics as small with prominent nuclei, while others were large cells, vacuolated and elongated, with intercellular spaces (Fig. 4c). The SEM image shows an apparently normal globular-shaped plantlet (Fig. 4d). The intense fluorescence by DAPI showed the presence of cells containing a high concentration of DNA, illustrating high mitotic activity which, subsequently, induced the formation of NCs (Fig. 4e).

3.2. Induction of NCs from leaf explants of *V. reitzii*

Leaf bases contained the intercalary meristem in the basal region (Fig. 1b, see arrow) and were grown in MSB medium supplemented with NAA (4 μM) plus BAP (2 μM), allowing the characterization of NCs induction in *V. reitzii* (Fig. 5). Morphological analysis indicated that NCs induction proceeded in a sequential manner, starting with swelling at the basal part of explants after the first week of inoculation (Fig. 5a). Through histochemical analysis, elongated cells were observed, and the reaction with TB-O showed acidic polysaccharides in the cell walls and cytoplasm (Fig. 5b). SEM analyses showed the formation

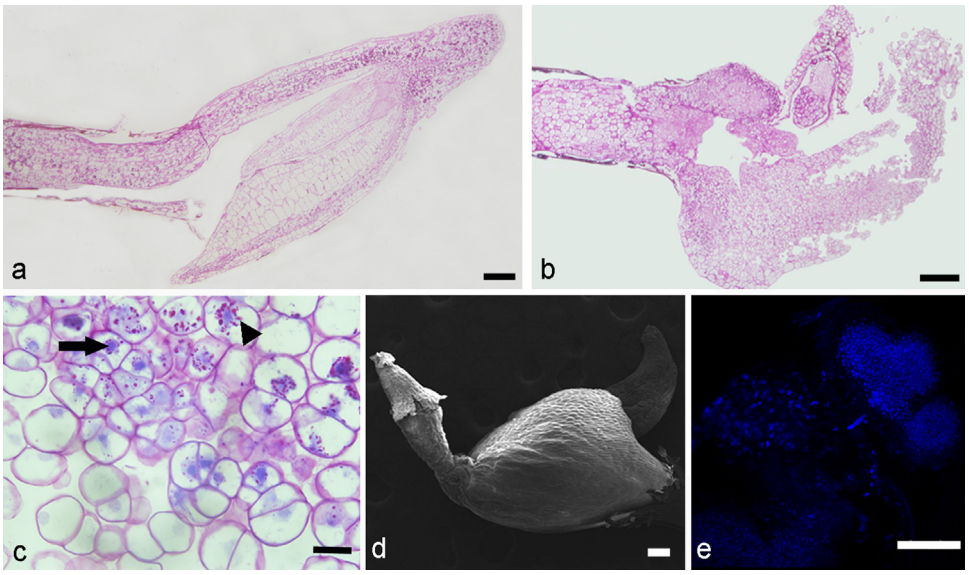

Fig. 4. Nodular cultures from seeds after 18 days in culture media with NAA (4 μM). (a) Longitudinal section of control stained with PAS test; (b) longitudinal section stained with PAS; (c) longitudinal section subjected to PAS + CBB. Blue-stained prominent nuclei (arrow) and red-stained starch granules (arrowhead); (d) SEM image; (e) CLSM image of longitudinal section of the root apex. Bars: (a, b, d, e) 200 μm; (c) 25 μm.

of protrusions at the base of the leaf explant and a high presence of trichomes in the upper portion of the explant (Fig. 5c). The tissue showed little differentiation at the leaf base, confirming the presence of intercalary meristem.

In the second week of culture, stereomicroscopic examination revealed the formation of protrusions arising from the basal parts (Fig. 5d). These projections are characterized by the proliferation of cells at the base of the explant, forming some denser regions (Fig. 5e). The formation of these protrusions at the base of the explant was also evident in the SEM images (Fig. 5f). The formation of new protrusions, occurred both at the base of the explant and in those previously formed (Fig. 5g–i), was observed in the third and fourth weeks of culture. Some cells were small with dense cytoplasm, and others were large elongated cells with intercellular spaces (Fig. 5j). CLSM analysis revealed higher fluorescence intensity at the base of the explant and also at the edge of the protrusions, indicating higher mitotic activity associated with the formation of new meristematic zones (Fig. 5k). After 4 weeks (Fig. 5l), histochemical analyses identified prenodular structures with protoderm (Fig. 5m) and the formation of a meristematic zone in the subepidermal region of the nodules (Fig. 5n). Images obtained by SEM and CLSM showed proliferation of NCs (Fig. 5o and p). Under CLSM, a higher fluorescence was observed at the base of the explant and at the newly formed meristematic zones (Fig. 5p).

3.3. Ultrastructure of NCs of Vriesea

Ultrastructural analysis of the newly formed NCs revealed the two cell types previously detected by light microscopy (Fig. 6). First, small cells (approximately 20 μm in length) without intercellular space had large nuclei and prominent nucleoli, dense cytoplasm and thin cell walls. Small lipid bodies, small vacuoles showing vacuolar fusion, mitochondria and chloroplasts with starch granules and plastoglobules were observed. However, the highly electron-dense cytoplasm made it difficult to visualize other cell components (Fig. 6a–d). Second, large cells (about 50 mm in length) had no meristematic characteristics, were less dense and highly vacuolated, with intercellular spaces, and some featured an elongated tubular shape (Fig. 6e–h). In these cells, the vacuolar content was highly electron-dense, but chloroplasts also revealed starch granules associated with the thylakoid system and plastoglobules in the plastid stroma (Fig. 6h).

4. Discussion

Seedling development was inversely proportional to the induction of NCs in *V. friburgensis* var. *paludosa* seeds. Similar results were found in other species of bromeliads cultivated in medium with different growth regulators. In *V. reitzii*, the highest percentages of NCs induction resulted from medium MSB liquid supplemented with 4 μM NAA (81.8%) or with 0.1 μM N-phenyl-N′-1,2,3-thidiazol-5-ylurea (TDZ) (80.9%) and when NAA was supplemented in the medium inhibits the normal germination of seeds [12]. For example, in seeds of *Tillandsia eizii* (Bromeliaceae), the normal growth of seedlings was inhibited in Knudson culture medium supplemented with NAA [25]. Moreover, inhibition of seed germination was followed by callus proliferation and induction of adventitious buds when inoculated into Knudson medium with BAP and NAA [26].

Rech Filho et al. [27] noticed the formation of greenish-yellow protrusions that also appeared in the basal region of the plantlets of *V. reitzii*. In *Dyckia distachya* (Bromeliaceae), the induction of a callus structure was also reported after

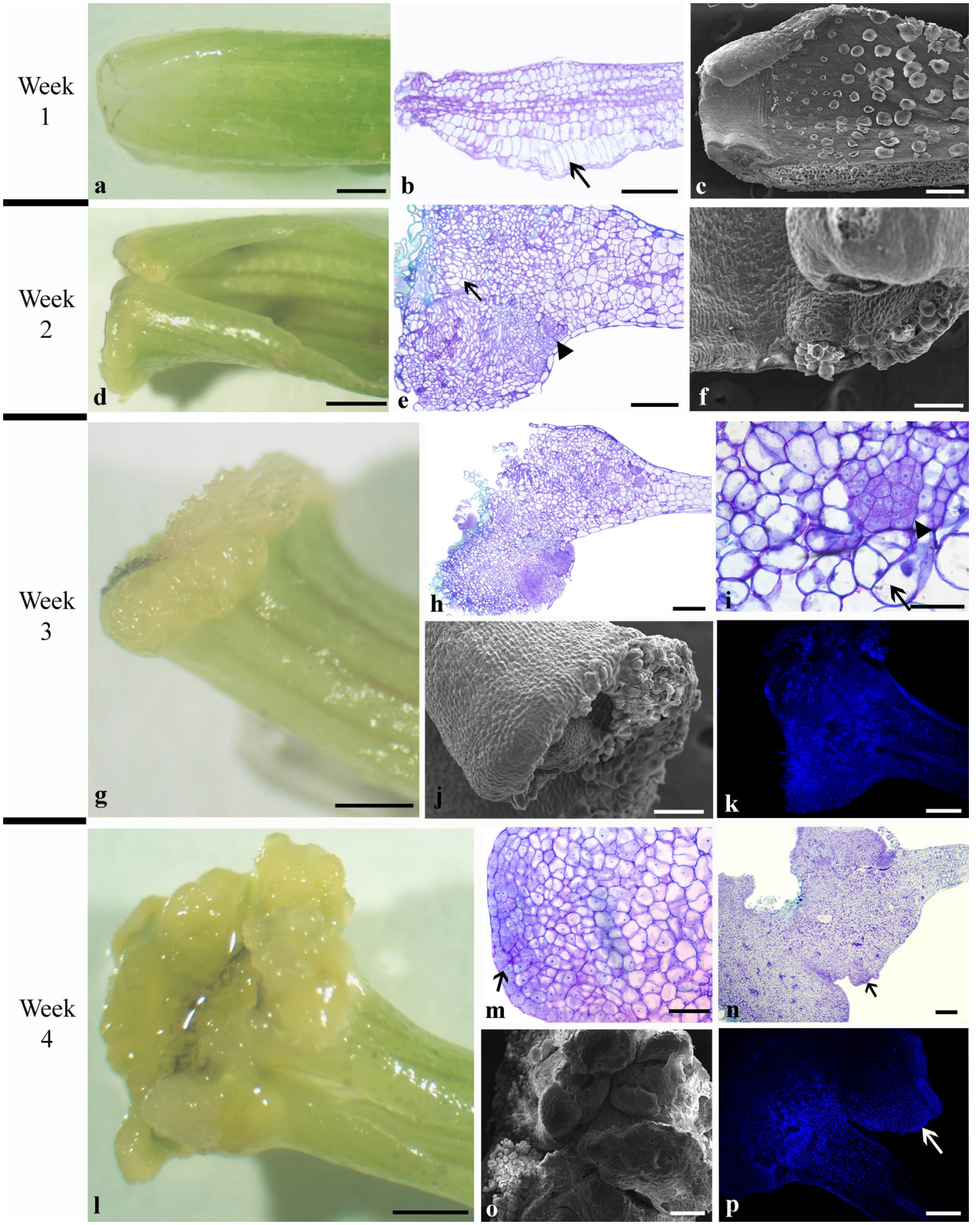

Fig. 5. Induction of nodular cultures from leaf explants of *V. reitzii*: (a) leaf explant segment after one week on culture medium; (b) longitudinal section subjected to the TB-O test. Elongated cells at the base of the explant (arrow); (c) SEM image; (d) formation of protrusions after two weeks on culture medium; (e) longitudinal section subjected to TB-O; (f) SEM image; (g) proliferation of nodular cultures in the third week of culture; (h) longitudinal section subjected to TB-O; (i) detail of cells. Small cells (arrowhead) and large cells (arrow); (j) SEM image; (k) CLSM image; (l) proliferation of nodular cultures after four weeks; (m) longitudinal section subjected to TB-O. Presence of prenodular structures with protoderm (arrow); (n) meristematic zones (arrow); (o) SEM image; (p) CLSM image. New meristematic zones (white arrow). Bars: (a, d, g, l) 1 mm; (b, c, e, f, h, j, k, n, o, p) 200 μm; (i, m) 50 μm.

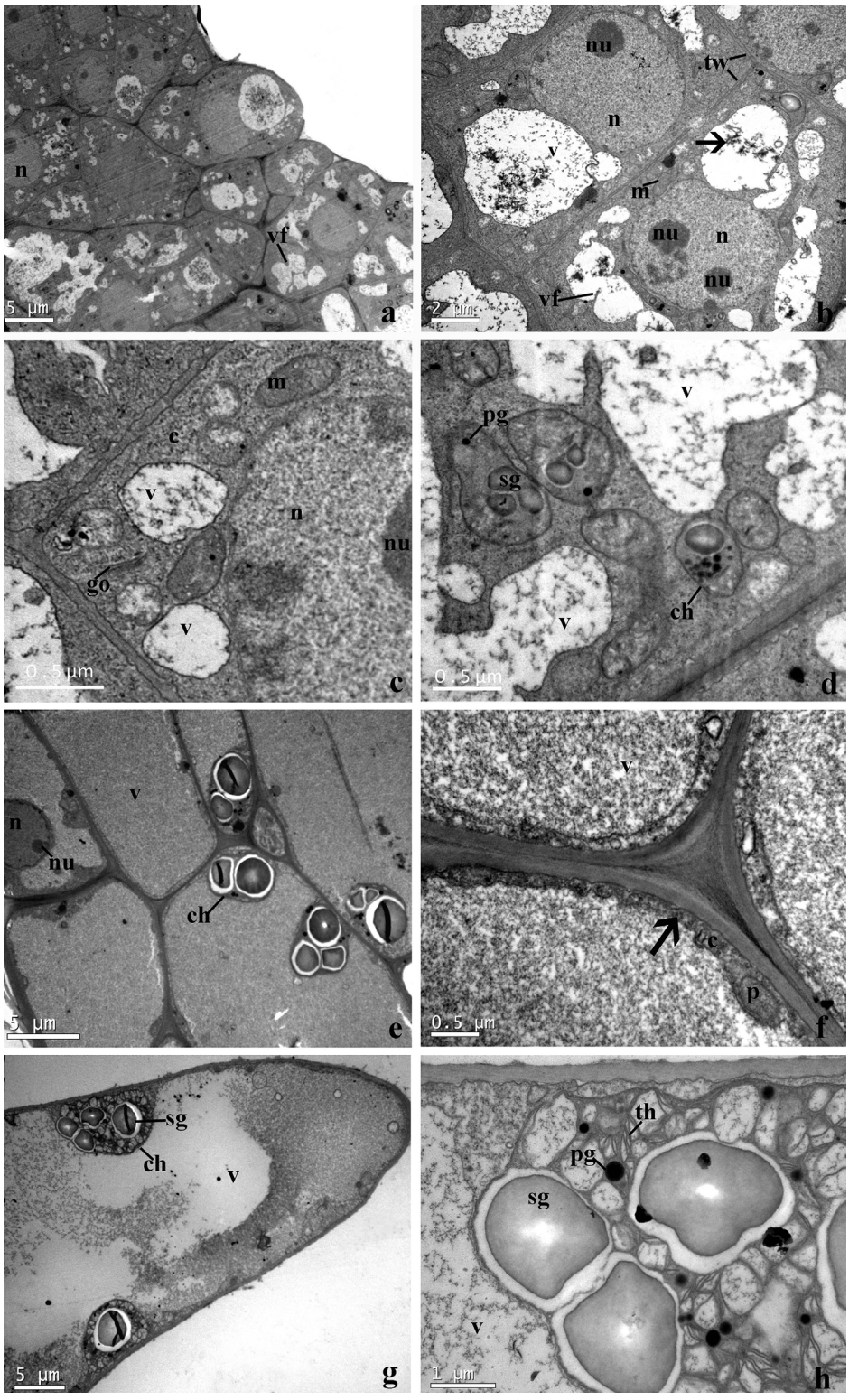

Fig. 6. TEM images of nodular cultures. (a–d) Ultrastructure of cells with meristematic characteristics. (e–h) Ultrastructure of vacuolated cells. (a) Cells without intercellular space and small vacuoles; (b) large nuclei with prominent nucleoli; vacuoles containing a highly electron-dense content (arrow); (c) mitochondria; (d) chloroplasts with starch granules and plastoglobules; (e) highly vacuolated cells; (f) reduced cytoplasm (arrow); (g) cells with a tubular shape; (h) detail of a chloroplast with starch granules and plastoglobules. c: cytoplasm; ch: chloroplast; go: golgi stack; n: nucleus; nu: nucleolus; m: mitochondria; p: plastid; pg: plastoglobules; sg: starch granule; th: thylakoid; tw: thin cell walls; v: vacuole; vf: vacuolar fusion.

plantlet formation [28], highlighting the fact that the root and shoot meristematic regions in bromeliads are responsive and usually promote the development of NCs. This phenomenon is probably related to the activity of competent cells, *i.e.*, cells able to respond to an external signal that activates a specific developmental pathway [29]. This competence is also associated with the juvenility of the tissue and organs of the explants [30–32].

In the present study, the higher equimolar effect of NAA relative to BAP was essential in promoting the induction of NCs. This fact highlights the key role of auxins in the processes of division, elongation and differentiation in plants [33] and, hence, the importance of auxins in controlling morphogenesis in *in vitro* cultures [34]. According to Beck [35], some of the expected responses associated with the action of auxins are the formation of primordia and development of leaves.

On the other hand, the induction of NCs from leaf explants of *V. reitzii* began with the formation of protrusions at the base of the leaf. Alves et al. [5], Dal Vesco et al. [6] and Dal Vesco and Guerra [3] also reported the formation of NCs in the basal parts of leaf explants in *V. reitzii*. The formation of similar structures in this region of the explant has also been observed in other species of bromeliads, including *Dickia macedoi* [36], *Neoregelia cruenta* [37], *V. friburgensis* var. *paludosa* [38], and *A. comosus* [7,39,40]. The tissue of the leaf base of monocotyledons, such as that found in the bromeliads, is less differentiated and has growth zones named intercalary meristems [41,42]. Therefore, the observed response in the induction of NCs in *V. reitzii* from leaf explants may be related to the presence of intercalary or marginal meristem, which would favor their regeneration competence. Firoozabady and Moy [39] tested different leaf sections (tip, middle and base) in *A. comosus*, and only the leaf base responded to the morphogenetic processes. These authors suggested that this response could be related to the proximity of the leaf base to intercalary meristem or the presence of rapidly dividing cells. Koh and Davies [43] achieved the micropropagation of bromeliads of the genus *Cryptanthus* using leaf explants containing intercalary meristems. Batista et al. [8] reported the induction of organogenic nodules in petiole segments of *H. lupulus* (Cannabaceae) from meristematic cells in the central area of vascularization. On the other hand, Alves et al. [5] induced NCs in leaf segments of *V. reitzii* and observed their development from parenchymatic tissue after three weeks of culture.

Histochemical analyses of NCs in different species of bromeliads allowed for the identification of meristematic zones composed of clusters of isodiametric cells with dense cytoplasm [3,4,7]. According to Dal Vesco and Guerra [3], the proliferation of NCs occurs through multiple formations of these meristems. In the present study, histological analyses of NCs originated from leaf bases and seeds showed the presence of this type of cell with meristematic features, which may be related to the subsequent formation of unipolar axis, as described by Dal Vesco and Guerra [3] and Dal Vesco et al. [4]. However, the proliferation of cells with meristematic characteristics could also lead to the formation of embryogenic structures [44], although bipolar structures were not observed in the NCs.

Ultrastructural analysis of NCs revealed cells with meristematic characteristics. These cells probably have repeated divisions, forming compact tissue without intercellular spaces. Similar ultrastructural features were found in cells in the meristemoids of plants during organogenesis [10,45]. Other cell types, highly vacuolated, with intercellular spaces in the NCs, may be related to the friability of these structures. Cells of the NCs presented starch grains in chloroplasts. According to Fortes and Pais [9], an accumulation of starch in prenodular structures during organogenesis in *H. lupulus* (Cannabaceae) results from the high energy requirement of this process. Thus, the presence of starch granules in the plastids found in dividing cells seems to be a typical feature of organogenesis [45].

Ultrastructural analysis of plants showing somatic embryogenesis reveals that small, dense cells with prominent nuclei are characteristic of embryogenic cells, whereas highly vacuolated cells are commonly found in clusters of non-embryogenic cells [10,46]. However, Firoozabady and Moy [39] induced somatic embryogenesis in *A. comosus*, producing embryogenic cell clusters containing small cells mixed with large, elongated and vacuolated cells. In orchids, Lee et al. [47] reported structures composed of compact and loose aggregates of cells, which then resulted in protocorm-like bodies. Nevertheless, meristemoids in *Bauhinia forficata* (Fabaceae) and *Glycine max* (Fabaceae) did not originate from typical meristematic cells, but rather from highly vacuolated cells, which divide unequally to produce different-sized daughter cells [45]. Therefore, the two cell types observed in the NCs are important to achieving high regenerative potential, culminating in the multiple production of adventitious shoots.

5. Conclusion

The concentrations and combinations of growth regulators determine the rate of induction of NCs from seeds. Thus, NAA (4 μM) added in the culture medium achieves a high induction rate, considering that the embryo first starts the germination process, and then the development of NCs takes place. Although NCs formed from seeds is related to root and shoot meristems, the formation from leaf explants is associated with the intercalary meristem. These meristematic cells are competent and generate an appropriate response in the induction medium, producing NCs by means of the proliferation of small cells with meristematic characteristics, as well as large vacuolated cells. These findings provide a better understanding of morphogenetic responses in bromeliads and, hence, the opportunity to develop optimized micropropagation protocols.

Conflict of interest

We have no conflict of interest to declare.

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