Exogenous auxin triggers callogenesis or rhizogenesis differentiation pathways in immature date palm leaf (*Phoenix dactylifera* L.)

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INTRODUCTION

Date palm is a dioecious perennial plant belonging to the *Arecaceae* family widely cultivated in arid regions of the middle East and North Africa. To develop date palm culture in sub-sahelian countries, where climate and soil conditions are more restricting, a program of micropropagation by somatic embryogenesis has been initiated. In monocotyledons, and particularly in Arecaceae family, indirect somatic embryogenesis through callus production is firstly required to produce *in vitro* plantlets. This first step requires auxin supplementation. However growth regulators as auxin were assumed to promote organogenetic pathways according to their nature and their concentration. . We propose to study the effect of Naphtalenacetic acid (NAA) on the induction of callogenesis or rhizogenesis. Histological studies were performed to assess that the (de)differentiation events and developmental commitment were correlated with auxin responses of competent cells.

MATERIAL AND METHODS

Immature leaves of 6 months-old plants were aseptised, then fragmented into 10 segments. Proximal segments were cultivated in medium described in Sané *et al.* (2006) supplemented with 1 µM or 54 µM of NAA.
Samples were collected from d0 to d33 of the culture then fixed, dehydrated in graded ethanol series, and finally included into Technovit resin. Semi-thin sections (3 µm) were double stained with PAS (periodic acid Schiff) for polysaccharides, and NBB (naphtol blue black) for soluble proteins (Buffard-Morel *et al.*, 1992).

Fig.1: Fragmentation into 10 segments of immature leaf.
Only the second and the third proximal segments were cultivated in presence of 1µM or 54 µM NAA (showed by circle).

RESULTS : COMMON INDUCTION PHASE.

At d0-d2, histological studies led us to establish that active cells, which were originated from the differentiating leaf tissue, did not exhibit any modifications (fig. 2A.). These cells were localised between the vascular parenchyma: mainly around phloem in minor vein, and around less diffrentiated-xylem in vascular bundle. In interfacial parenchyma, we observed newly reactivated-cells (d2-d5), and the phenomenon propagated in a centrifuge manner (fig. 2B, 2C.). Such histological events were common whatever the pathway induced (callogenesis with 1 µM NAA, or rhizogenenis with 54 µM NAA). Newly reactivated cells appeared only in vascular parenchyma. Such cells exhibited specific cellular characters such as a lobed nucleus, prominent nucleolus, fragmented vacuome, dense cytoplasm with proplast and reticulum (fig. E, F, G)

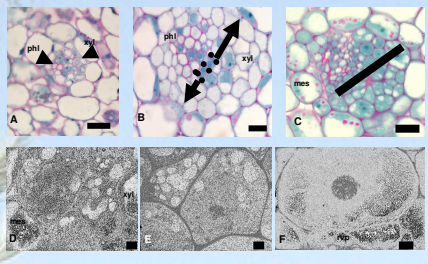

Fig.2: Histological events during the induction phase of callogenesis or rhizogenesis in immature date palm leaf cultivated *in vitro* (d0 to d7). **A**: In minor vein, dense-cytoplasm cells are still present in vascular parenchyma. They are probably at the end of their differentiation as the leaf segment was differentiating at d0. **B**: New reactived cells appeared in the interfacial parenchyma, then the reactivation propagates in a centrifuge manner. **C, D, E, F**: Recruitment phase of reactivated cells from the vascular parenchyma. Three types of cells are studied by transmission electronic microscopy along a virtual line corresponding to the diameter of the minor vein: reactivated cell near mesophyll (D), zone of the interfacial parenchyma (E) and fully reactivated cell (F). These cells exhibit usual cellular characters of a reactivated cell as described in the literature. mes: mesophyll cell; phl: phloem; rvp: reactivated vascular parenchyma; xyl: xylem A, B, C: bar= 20 µm. D, F: bar = 2 µm. E: bar = 0.5 µm.

[ANA]

CALLOGENESIS PATTERN.

Callogenesis was induced by 54 µM NAA. Active mitosis were observed (fig. 3A, 3B) and led to obtain bipolar clusters of dense cells (fig. 3C). These cells exhibited calloïd shape according to Nyman *et al.* (1983). A primary nodular callus was fully differentiated into three concentric cell zones since d28 (fig. 3D, 3E). Few amyloplasts were rarely observed in the peripheric layer 3 (fig. 3F). Since d28, growth proceeded with anticlinal division (fig. 3F). Numerous primary nodular callus were emerging through leaf tissue since d63 (fig. 3G).

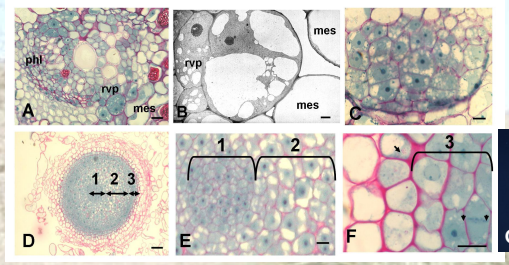

Fig. 3: Callogenesis pattern. **A, B, C**: **Proliferation phase (d9-d12)**. Only reactivated cells located on both sides from the interfacial parenchyma re-enter in division (A, B) then in active mitosis (C). The other cells as reactivated vascular parenchymatous cell (rvp) and mesophyll cells (mes) rarely show division. At th end of the proliferation phase, cells exhibit calloid shape (central voluminous nucleus, dense nucleolus, star-shape cytoplasm). **D, E, F**: **Structuration and differentiation phases (d14-d28)**: nodular callus shows three zones (D). Internal zone (zone 1) is constitued of small meristematic cells and zone 2 has cortical vacuolated cells (E). The peripheric zone (zone 3) exhibits rectangular dense cells where anticlinal division could appear to favour radial growth (arrow heads). Numerous layers of optically empty cells bordered with purple cell wall surround the callus. Few amyloplasts can be stained with PAS in such a cell (arrow). **G**: After 9 weeks (d63), primary nodular calluses appear and emerge from the leaf tissue. A, C, F: bar = 20 µM. E: bar = 10 µm. D: bar = 50 µm. B: bar= 2 µm.

RHIZOGENESIS PATTERN.

Rhizogenesis was induced by 1 µM NAA, active mitosis were observed (fig. 4A to 4D) and a meristematic clump was growing (fig. 3D). Then it became well characterized by the columella differentiation. During active proliferation, cells progressively acquired meristematic characters and appeared small and dense. Phloem and xylem traces moved away. When a certain distance was reached, the columella started to differentiate at the opposite pole of vascular tissue. The meristematic clump still showed vascular connection with the vascular bundle from the leaf explant (fig. 4E, 4F). Numerous large amyloplasts were observed along periclinal columella cell walls (fig. 4G, 4H). Fully developped root or racinoid were observed since d63 (fig. 5).

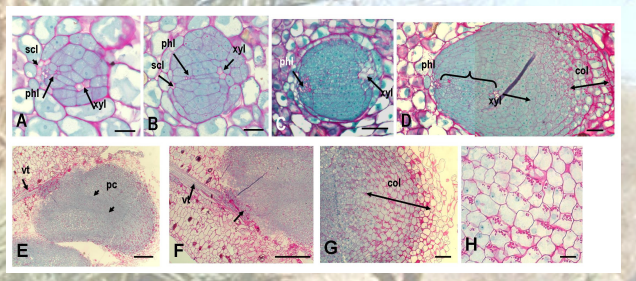

Fig. 4: Rhizogenesis pattern. **A to C**. **Proliferation phase (d9-d12)**. Only reactivated cells from vascular parenchyma entered in active mitosis. As proliferation occurred, the distance between phloem and xylem increased. Cells became more and more small and exhibited a very dense cytoplasm. **D**: **Structuration phase (d12-d14)**. When a certain distance is reached (60 µm), the clump continues to proliferate at the opposite pole. Cells showing purple cell wall and organelles are easily distinguishable. **E, F (d33)**: Such a root meristematic clump is still attached to the vascular tissue (vt) from the leaf explant. It shows denser cells in the centre which could further differentiate in procambium (pc) (longitudinal sections). **G, H**: **Differentiation of the statenchyme(d14-d33)**: cells with numerous large amyloplasts designated as statocytes differentiate and are characteristic of a columella (longitudinal sections). A, B, C, D, H: bar = 20 µM. E, F: bar = 100 µm. G: bar = 50 µm.

CONCLUSIONS AND PERSPECTIVES

Exogenous NAA could trigger developmental pathways but they presented a common induction phase. The same vascular parenchymatous cells were implicated in such cell reactivation. Then, since d14, the proliferation phase led to two kind of cells: calloïd and meristematic according the pathways undergone. Structuration and Differentiation occurred later (since d14). Multicellular structures produced were completly distinct and showed classical histological shapes of callus or root/racinoïd as described in literature.

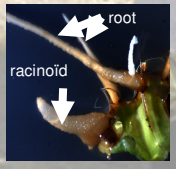

Fig. 5: Root and racinoïd (d63). Two types of root-like structures developped from root meristematic clump. Typical roots were easily distinguished as they were fine whereas the other organ showed glove-finger shape. Histological studies (data not shown) revealed normal cortical parenchyma and actinostele (M. Collin, IRD, personnal communication).

The fact that only vascular parenchymatous cells were identified as competent ones could be linked to their differentiation status, their proximity with vascular tisssue (sieve), and probably the endogenous level of auxins. Callogenesis seemed to be associated with the position of the segment in the immature leaf (B. Gueye, personnal communication). It was also established that exogenous auxins could be recognized by efflux transporter as PIN and that such PIN could be redistributed under exogenous auxins during *in vitro* culture (Benkova et al., 2003). Such an effect on endogenous auxin has already been described in alfalfa (Pasternak *et al.*, 2002).The common induction phase and the calloïd type cells led us to question about the reversibility and the switch or determination (fixation ?) of the developmental pathway. Moreover, it would be very interesting to investigate the efflux/ influx transport, auxin gradient and the diffusion of such exogenous auxins during the induction phase (2,4-D by example as antibodies are available).

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