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Short note

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Observation of unexpected neo like-fruit development from *Cakile maritima* calli

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Abstract: Parthenocarpy, the ability of some plants to undergo fruit growth in absence of fertilization, is an important question of basic science and the subject of much interest due to its possible agricultural benefits. In the context of our cellular biology studies on a halophyte of interest, *Cakile maritima,* we generated calli, pluripotent cell masses, that unexpectedly allowed the appearance of parthenocarpic fruits without any floral tissues. These observations raise the hope to develop an *in vitro* model to study parthenocarpic fruit development.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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1. Introduction

For several years, we are studying *Cakile maritima*, a promising model of halophyte in a worldwide context of increasing salinization of lands due to climate change (Arbelet-Bonnin *et al*., 2019). In addition to whole plant studies (Debez *et al*., 2006; Ellouzi *et al*., 2014; Ben Hamed-Louati *et al*., 2016 b; Arbelet-Bonnin *et al*., 2020) we underwent cellular biology studies (Ben Hamed-Laouti *et al*., 2016 a; Arbelet-Bonnin *et al*., 2018) for which we have developed a *C. maritima* cultured cell suspension that starts by calli generation as a first step (Ben Hamed *et al*., 2014). It is now admitted that *in vitro* cultured cells do not undergo a full dedifferentiation but rather a transdifferentiation, leading to increased developmental potency and/or cell proliferation (Sugimoto *et al*., 2011; Fehér, 2019). The supposed totipotency capacity of plant cells (Haberlandt, 1902) is largely operated for the *in vitro* culture of plants. Calli are thus frequently considered as transient tissue dedicated to somatic embryogenesis allowing the plant regeneration (Sugimoto *et al*., 2011; Fehér, 2019). Interestingly, hormonal balances play an important role in many developmental processes in plant (Molesini *et al*., 2020), and in particular the balance between auxin and cytokine seems to be crucial during *in vivo* parthenocarpic fruit development (Pandolfini, 2009; Joldersma and Liu, 2018; An *et al*., 2020; Sharif *et al*., 2022).

Parthenocarpy is the ability of some plants to undergo fruit growth in absence of fertilization. Parthenocarpy has contributed to some of humanity's domestication of plants such as breadfruit or banana (Zerega *et al*., 2004; Kislev *et al*., 2006; Sardos *et al*., 2016). Furthermore, it represents a highly desirable trait in agronomy since seedless fruits are highly appreciated by consumers and fruit set is less affected by environmental factors in absence of fertilization (Ruan *et al*., 2012). Therefore, parthenocarpy is the subject of much interest and leads to many studies (Sharif *et al.*, 2022). However, to our knowledge, all studies published so far have been conducted in whole plants but not at the level of cell cultures so far.

We report here the first appearance of neo-like fruits directly on calli obtained and long maintained on callus-inducing medium (CIM). These observations raise the hope to develop a cellular model that will allow *in vitro* studies of parthenocarpic fruit development.

2. Materials and Methods

Plant material and establishment of calli

Cakile maritima seeds used in this study come from Raoued (North of Tunisia). The seeds cultivation *in vitro* conditions were established as described by Ben Hamed *et al*. (2014). Briefly, bleached seeds were placed in petri-dishes containing Murashige and Skoog medium (1962) hormone-free (MS, Sigma), supplemented with 30 $g.L^{-1}$ sucrose, 8 $g.L^{-1}$ agar and the pH was adjusted to 5.8. This medium induced seed germination under a light cycle of 12 h light and 12 h dark with 40 $\mu E\ m^2\ s^{-1}$ at 22°C. The stems of 14-days-old seedlings were then chopped finely and placed on an agar-callus-induced medium (CIM) (Valvekens *et al.*, 1988) and then put in a growth chamber, in the same conditions that for seed germination. CIM medium contain 6.2 $g.L^{-1}$ Gamborg B5 (Gamborg *et al.*, 1968) from Sigma supplemented with 20 $g.L^{-1}$ glucose, 8 $g.L^{-1}$ agar and phytohormones : cytokinin and auxin (Valvekens *et al*., 1988; Akama *et al*., 1992). The precise hormone balance depends of plant species (Pacheco *et al.*, 2012; Thomas and Hoshino, 2015). For *C. maritima* we used 9.06 µM of 2,4-dichlorophenoxyacetic acid (2.4 D) and 0.46 µM of kinetin (Kn) that were shown to be efficient (Ben Hamed *et al*., 2014). The pH was adjusted to 5.7. After two weeks, calli were formed from the fragments of seedlings tissues. When the size of the calli reached 1 cm in length, they were subcultured on a fresh CIM medium.

For some experiments, the hormonal balance was modified with 9 µM of Kn and 2-4D, or 0.46 µM of Kn and 2-4D and calli were grown during two months. To assess the putative role of volatile organic compounds (VOCs) emitted from mature fruit, a surface sterilized fruit of *C. maritima* was added in petri-dishes with calli during 2 months.

3. Results and Discussion

Cakile maritima calli were subcultured every four weeks on the CIM medium, otherwise they began to brownish and lose their ability to be sub-cultured. During a callus subculture, a spontaneous green excrescence from calli easily visible (Fig. 1a) was observed. As the subcultures went along on fresh medium CIM, green excrescences grew on calli and more excrescences appeared on different calli (Fig. 1b and 1c). Each appeared excrescence grew all-long the time (Fig. 1d).

After 58 days, numerous green structures resembling *C. maritima* differentiated tissues arise (Fig. 2). 40% of them are clearly green differentiated tissue although we cannot identify specific organ structures (Fig. 2A, B). On the contrary, the other 60 % of green differentiated structures resemble *neo* like-fruits (Fig. 2C-E). Indeed, they present two asymmetric segments reminiscent of the typical dimorphic fruit that

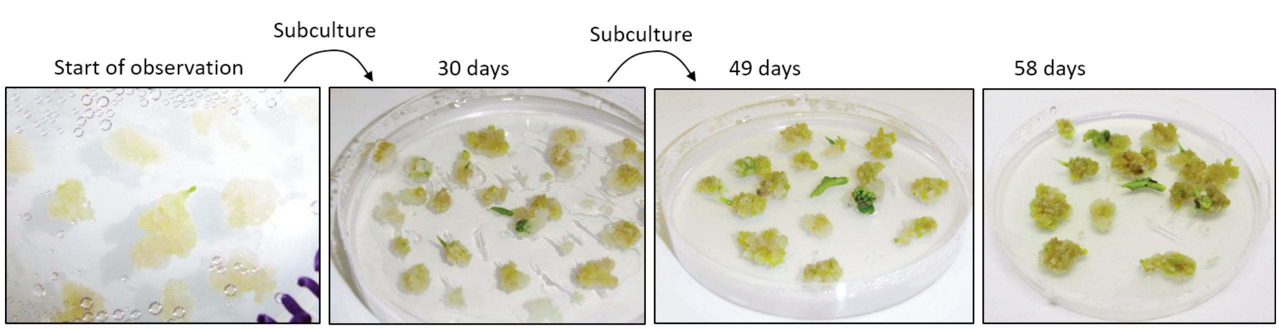

Fig. 1 - Emergence and development of parthenocarpic fruits on *Cakile maritima* callus. First observation (a), observations after 30 days (b); 49 days (c) and 58 days (d).

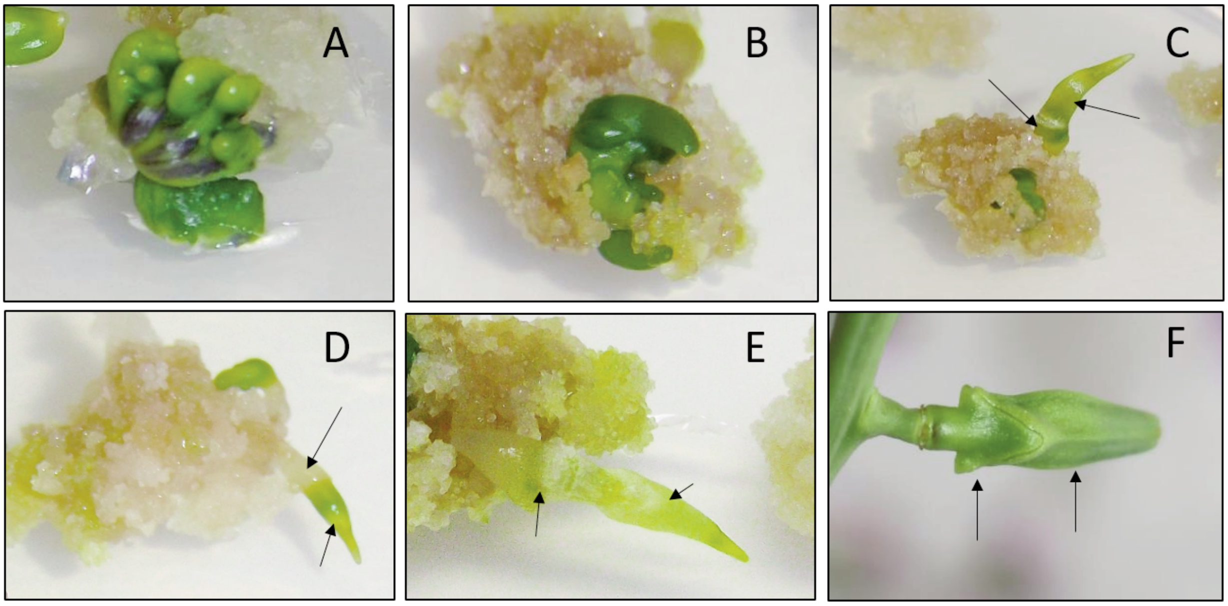

Fig. 2 - Various green structures observed on *Cakile maritima* callus. A-B. Undefined differentiated green structures. C-E. Typical dimorphic fruit like structures. F. Typical dimorphic fruit developed on a whole plant of *C. maritima* (arrows indicated the two asymmetric segments).

develops on whole plant *of C. maritima* (Fig. 2F). These *neo* like-fruits did not contain seeds and are thus parthenocarpic. Such parthenocarpic fruits have never been reported in *C. maritima* in the literature. More interestingly, this represents not only the first report of parthenocarpic fruits forming from calli, but also forming from non-floral tissues. These parthenocarpic fruit appeared without any change in the hormone balance, a mean generally used to triggered shoot or root regeneration *in vitro* in the context of somatic embryogenesis and plant multiplication (Thomas and Hoshino, 2015; Das *et al.*, 2018; Shin *et al.*, 2020).

Parthenocarpy can be artificially obtained by applying synthetic growth factors to unpollinated ovaries (Pandolfini, 2009; Molesini *et al*., 2020). Auxins seem to play a prominent role in triggering and coordinating the transition from flower to fruit, and exogenous supplies of auxins to unpollinated flowers could induce fruit growth in various plants, suggesting that these hormones can replace the signals provided by pollination and fertilization (Pandolfini, 2009; Molesini *et al*., 2020). Cytokinin could also induced parthenocarpy but probably through modulation of auxin metabolism (Molesini *et al*., 2020; Sharif *et al.*, 2022). We tried to modify the hormones concentrations and the balance between Kn and 2-4D by using 0.46 µM or 9 µM of both of these hormones but unfortunately, no new spontaneous green excrescences development were observed on the 20 calli present on each petri dish (n=2 per conditions). Since we observed a multiplication of these parthenocarpic fruits in the same petri-dishes, we asked ourselves if this phenomenon could be reminiscent of fruit ripening triggered by volatiles organic compounds (VOCs) such as ethylene released from already developed fruits (Tohge *et al*., 2014). Even if the role of ethylene still appeared unclear (Sharif *et al.*, 2022), ethylene responses could also lead to parthenocarpic fruit development (Pandolfini, 2009). We thus put a mature *C. maritima* fruit harvested from a fully developed plant in a petri-dish with calli, as a putative VOCs furnisher to calli. One more time no excrescences appeared on the 20 different calli present in the petri dish. Although we have no clear explanation at the moment, the question of genetic homogeneity of callus cells is not solved and only certain cells of a callus could be regarded as totipotent and thus involved in organ regeneration (Fehér, 2019). Moreover, this ability to develop parthenocarpic fruits disappeared after three months and more subcultures. This suggests that only freshly prepared calli are able to develop parthenogenetic fruits. Accordingly, if callus tissue can express a wide variety of genes especially at the early phase of their development, their transcriptome seems to be homogenize along time (Fehér, 2019).

Although we cannot control the appearance and development of parthenocarpic fruits on calli at this time, these data certainly, deserve more investigations. Recent genomic studies have greatly contributed to elucidate the role of phytohormones in regulating fruit initiation, providing at the same time genetic methods for introducing seedlessness in horticultural plants. Moreover, that some plants may produce fruit without the need for fertilization by the male gamete remains an important question of basic science. Therefore, the development of an *in vitro* model of parthenocarpic fruits will certainly be an essential tool to understand how a fruit could form without the need of fertilization nor floral tissues.

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