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Callus induction and establishment of cell suspension cultures

of the halophyte *Armeria maritima* (Mill.) Willd.

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Abstract: The objective of this work was to establish a cell suspension culture from *Armeria maritima* (Mill.) Willd., an halophyte so far only valuable for horticultural purposes, as a pre-requisite step towards the production of bio-actives secondary metabolites *in vitro*. For the initiation of callogenesis, five types of explants were tested: leaves from mature plants; leaves, cotyledons and roots from axenic seedlings; seeds. Three criteria either from a quantitative (percentage of callus induction) or a qualitative basis (aspect and friability) were used to evaluate the effectiveness of callus formation. Seeds-derived calli grown in the dark on MS medium supplemented with 4.5 μ M 2.4D and 0.93 μ M KIN were successfully selected for the establishment of cell suspension cultures, characterized by a growth index of 3.4 after 14 days of culture.

Keywords: *Armeria maritima* ; callus induction ; cell suspension ; halophyte ; explant ; plant growth regulators

1. Introduction

Halophytes are characterized by their ability to grow under saline stress, with concentrations of NaCl around 200 mM which is toxic to most crop species, leading to hyper osmotic shock and oxidative stress, nutrient imbalance, as well as to the inhibition of cell division and photosynthesis (Aslam et al., 2011). Salt tolerance in plants is determined by numerous physiological and biochemical processes, acting at molecular, cellular or morphological plant levels (Duarte et al., 2013). As an illustration for their adaptation to saline environment, halophytes produce osmo-protective compounds with quaternary ammonium or specific sugars such as glycine betaine and mannitol, respectively (Hanson et al., 1994), as well as polyphenols, in order to fight against oxidative stress notably caused by NaCl and UV radiations (Ksouri et al., 2007 and 2013; Rodrigues et al., 2015; Stankovic et al., 2015). This molecular adaptation towards antioxidant capacity makes these halophytic plants particularly interesting as a raw material for bioactive molecules, with multifaceted potential for either medical, cosmetical or nutritional applications (Buhmann and Papenbrock, 2013; Lopes et al., 2016; Medini et al., 2015).

Moreover, their adaptive mechanisms to salt stress often confer tolerance to other toxic elements such as heavy metal ions. Therefore halophytes are gaining an increased interest for their potential in phytoremediation of heavy metals contaminated soils (Van Oosten and Maggio, 2015). Halophytes also appear as a possible alternative to glycophytic crops in the growing environmental context where salinization of soils increasingly contributes to land degradation worldwide, thus highlighting the need to develop saline agriculture (Akinshina et al., 2016; Panta et al., 2014).

Armeria maritima (Mill.) Willd., also called “sea thrift” or “sea pink”, is a common halophytic plant growing in occidental coasts of northern Europe, actually used for horticulture purposes,

and already studied for its copper tolerance (Brewin et al., 2003). *A. maritima* belongs to the Plumbaginaceae family which is well-known for its halophytic genus *Limonium* (Hanson et al., 1994; Medini et al., 2015; Rodrigues et al., 2015). In the past, previous study described the characterization of flavonoids content in leaves of *A. maritima* (Lauranson et al., 1995). Flavonoids are well-known to have bioactive properties, especially interesting for cosmetic and pharmaceutical applications (Brand-Garnys et al., 2007). Moreover, Kumarasamy et al. (2002) showed an antibacterial effect of a methanol extract from *A. maritima* seeds against *Staphylococcus epidermidis*. Thus *A. maritima* could be considered as a multipurpose raw material.

Furthermore, the marine environment is a fragile ecosystem and the intensive harvest of wild seashore plants is not sustainable. Moreover, plants grown in field are submitted to seasonal and climatic fluctuations, inducing metabolic variations (Lauranson et al., 1995; Verma and Shukla, 2015) unsuitable with industrial valorization which needs standardized plant raw material. Thus, plant cell cultures of *A. maritima* could represent a suitable tool to supply high-value bioactive molecules, as it was already described for numerous other plants (Arias et al., 2016; Dias et al., 2016; Szopa et al., 2017). The main objective of this work was to establish an efficient protocol for friable callus induction and multiplication of *A. maritima* as a source for initiation of cell suspension culture, which is the first step towards *in vitro* metabolites production.

2. Material and methods

2.1. Plant material

Seeds of *A. maritima* var. *splendens* were purchased from Garden Seeds B.V. (Postbus 296, 1600 AG, Enkhuizen, Holland). For establishment of axenic seedlings, seeds were surface-

sterilized for 5h with agitation in 0.5% (v/v) Plant Preservative Mixture (PPM™, Plant Cell Technology), then washed three times in water and kept for 24h at 4°C prior to sowing on MS medium (Murashige and Skoog, 1962) at pH adjusted to 5.8, containing 3% (w/v) sucrose, solidified with 0.8% (w/v) agar and sterilized by autoclaving for 15 min at 121°C. Seeds were incubated for 6 weeks in a growth chamber at 25°C under a continuous photosynthetic photon flux density (PPFD) of 50 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ provided by cool-white fluorescent lamps.

A. maritima var. *splendens* plants cultivated in pots were purchased by Le Jardin du Pic Vert (26 Carrière Dorée, 59310 Orchies, France).

2.2. Callus induction

Five types of *A. maritima* explants were used for callus induction: 1 cm segments of leaves from mature plants cultivated in pots (Fig.1a); seeds (Fig.1b); young leaves, cotyledons and roots from 6 weeks-old axenic seedlings (Fig.1c). Leaves from plants grown in pots were first washed in ethanol for 30 sec, then surface-sterilized for 5 min with sodium hypochlorite (3.2% active chloride) containing 0.1 % (v/v) Polyoxyethylene (20) sorbitan monolaurate (Tween 20), and washed three times with sterile deionized water. Seeds were sterilized as previously described (§2.1).

Explants were inoculated in Petri dishes on MS medium including vitamins, supplemented with 3% (w/v) sucrose and growth regulators. Two auxins were tested: 4.5 μM 2,4-dichlorophenoxyacetic acid (2.4D) or 2.7 and 5.4 μM α -naphthaleneacetic acid (NAA), as well as two cytokinins: 0.93 μM kinetin (KIN) or 0.4 μM 6-benzyladenine (BA). The pH was adjusted to 5.8 and media were solidified by 0.8% (w/v) agar before autoclaving for 15 min at 121°C. Cultures for callus induction were maintained in a growth chamber at 20°C in the dark during eight weeks.

2.3. Cell suspension culture

Cell suspension cultures were established with 5g of friable seed-derived calli, transferred to a 250 mL Erlenmeyer flask containing 50 mL of MS liquid medium, supplemented with 4.5 μ M 2,4D, 0.93 μ M KIN and 3% (w/v) sucrose. The pH was adjusted to 5.8 before autoclaving for 15 min at 121°C. Cell suspension cultures were incubated at 20°C on a rotary shaker (130 rpm, 20 mm range) in the dark. Cells were first sub-cultured after 14 days by inoculating cell suspensions into fresh medium (1:2, v/v). For further subcultures, higher dilution was performed (1:4, v/v), in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium. For the establishment of growth curves, 14 days old inoculum culture (approximately 4g fresh weight) was used in 100mL flasks containing 40 mL of medium. Cell growth was determined at 3-4 days intervals during a 24 days period, by measuring the fresh weight of cells in the pellet after 10 min centrifugation (4.000 rpm), then by measuring the dry weight after freeze-drying of cells. All measures were performed at least in triplicate.

2.4. Statistical analysis

For callus induction, 40 explants were used for each experiment, and each experiment was performed in triplicate. For cell suspensions characterization, each growth measurement was repeated thrice, and experiments for statistical analysis were performed at least in triplicate. Data were subjected to analysis of variance and means were compared, with ANOVA or Kruskal-Wallis (depending on the results of Shapiro test for normality and Bartlett test for homogeneity of variance) and Tukey or Dunn test, respectively (R software).

3. Results and discussion

3.1. Effects of explant type on callus induction

Callus formation in *A. maritima* is dependent on explant type (Table 1) as it was already reported for many plants (Farhadi et al., 2017; Pacheco et al., 2012; Veraplakorn, 2016; Yan et al., 2009). Leaves from mature soil-grown plants and axenic seedlings (Fig1 d-e) , as well as roots from axenic seedlings, showed the best results, with a mean of 43.1 to 47.5% of explants forming calli after 8 weeks of culture. Seeds showed only 20.8% callus induction, while cotyledons exhibited a tiny callus induction (0.8%). This last result is in disagreement with Aly et al. (2002a and b), who reported 28.6% callus induction from cotyledons from *A. maritima*. The only differences between these two studies are the development stage of the axenic seedlings used as raw material (5 days-old *versus* 6-weeks-old in our study), and vitamins content in the MS medium for callus induction (we used MS vitamins instead of B5 vitamins). Thus, the capacity for callus induction in *A. maritima* was low compared with the halophytes for which *in vitro* culture protocols are already reported in the literature, with callus formation reaching 89% for several *Limonium* species (Aly et al., 2002a and b) and 94% for the Malvaceae *Kosteletzkya virginica* (Ruan et al., 2009).

Despite their relatively poor callus formation capacity, the seeds were a very interesting raw material for callus induction: only one medium was needed for the one-step protocol, and they allowed the formation of friable calli, which is a prerequisite for the establishment of cell suspension cultures. Germinating seeds produced callus along the length of the collar from the elongated seedling (Fig.1g). After 8 weeks, calli were excised from the explant and transferred to a fresh medium. After a period of 2-3 weeks, friable light yellow areas were developing on the brown primary calli (Fig. 1h). These light yellow areas were removed after 3 weeks and cultured separately to generate homogenous callus line (Fig.1i) further used for initiation of cell suspension. Calli induced from *A. maritima* leaves- and roots- derived explants were more compact and darker in spite of subcultures (Table 1), so they were not selected for further initiation of cell suspension culture. Moreover, seeds allowed a stable sourcing, and were easier

to sterilize before *in vitro* culture than mature soil-grown *A. maritima* plants, which were subjected to high contamination level. In most cases of plant callogenesis described in the literature, callus formation is generally initiated from leaf or stem explants; few studies reported the use of seeds as explant for callus initiation (Afolayan and Adebola, 2010; Akashi et al., 1993; Bernabé-Antonio et al., 2015; Straub et al., 1988; Yuan et al., 2009). Our results confirm the interest for this innovative plant culture experimental procedure, allowing a one-step direct callus induction.

3.2. Effects of growth regulators on callus induction

For further protocol optimization, seeds, leaves and roots from axenic seedlings were placed on MS medium with different growth regulators concentrations already described by Brewin et al. (2003). 2.7 or 5.4 μM NAA and 0.4 μM BA were tested as auxin and cytokinin instead of 4.5 μM 2.4D and 0.93 μM KIN. From the strict point of view of callus induction capacity, media containing NAA and BA were at least as effective than medium supplemented with 2.4D and KIN (Table 2), regardless of the type of explant. For example, MS medium supplemented with 2.7 μM NAA and 0.4 μM BA induced a 27.5 % callogenesis efficiency from seeds, instead of 18.3% with a 4.5 μM 2.4D and 0.93 μM KIN hormonal balance. But in the presence of NAA and BA, numerous roots developed from compact organogenic calli (Fig.1f). This was incompatible with our goal, aiming at the induction of friable calli, the most suitable material for further cell suspension initiation. This high capacity of the growth regulators NAA/BA to work in tandem for inducing root differentiation was already described (Veraplakorn, 2016).

According to the rate of friable calli development, our optimal procedure consists on the primary induction of calli from sterilized seeds grown on MS medium supplemented with 4.5 μM 2,4-D, 0.93 μM KIN, and sucrose 3% (w/v), incubated 8 weeks in the dark at 20°C, and

followed by subcultures from light yellow friable areas, until the establishment of an homogeneous callus line.

3.3. Initiation and growth of cell suspension culture

Light yellow parts of calli from seed explants grown in MS medium supplemented with 4.5 μM 2,4-D and 0.93 μM KIN were sub-cultured every 6 weeks in a fresh medium, until obtaining an homogenous callus line, which was used for the establishment of cell suspension cultures. The growth curve of *A. maritima* cell suspension, based on fresh and dry weight quantification, is represented in Fig.2. Cell suspensions entered the exponential phase after 3 days of culture, until the 14th day, with a growth index of 3.4 and 1.9 on a fresh and dry weight basis, respectively (growth index of the culture = (final weight – initial weight) / initial weight). The comparison of these growth parameters with those reported for other plant cell suspensions (Ahlawat et al., 2017; Pacheco et al., 2012) demonstrates that this *A. maritima* cell suspension is healthy and well-adapted for further investigations towards metabolite production. After the stationary phase (from day 14 to day 17), cultures showed a decrease in cell density. Suspension cultures were maintained for 2 months with a reproducible growth pattern.

In conclusion, an efficient cell suspension culture was established with seed-derived friable calli of *A. maritima*, as a prerequisite for further *in vitro* studies on the production of bioactive specialized metabolites. Calli with different morphological characteristics were obtained from other explants (axenic seedlings, leaves), and could be further used for micropropagation experiments. Our work is a preliminary step for the bioactive molecules production by plant cell culture, which correlates with the increasing interest of the scientific community towards topics at the interface of plant biotechnology and phytochemistry (Ahlawat et al., 2017; Dias et al., 2016; Mustafa et al., 2011; Pacheco et al., 2012; Shubashini et al., 2014).

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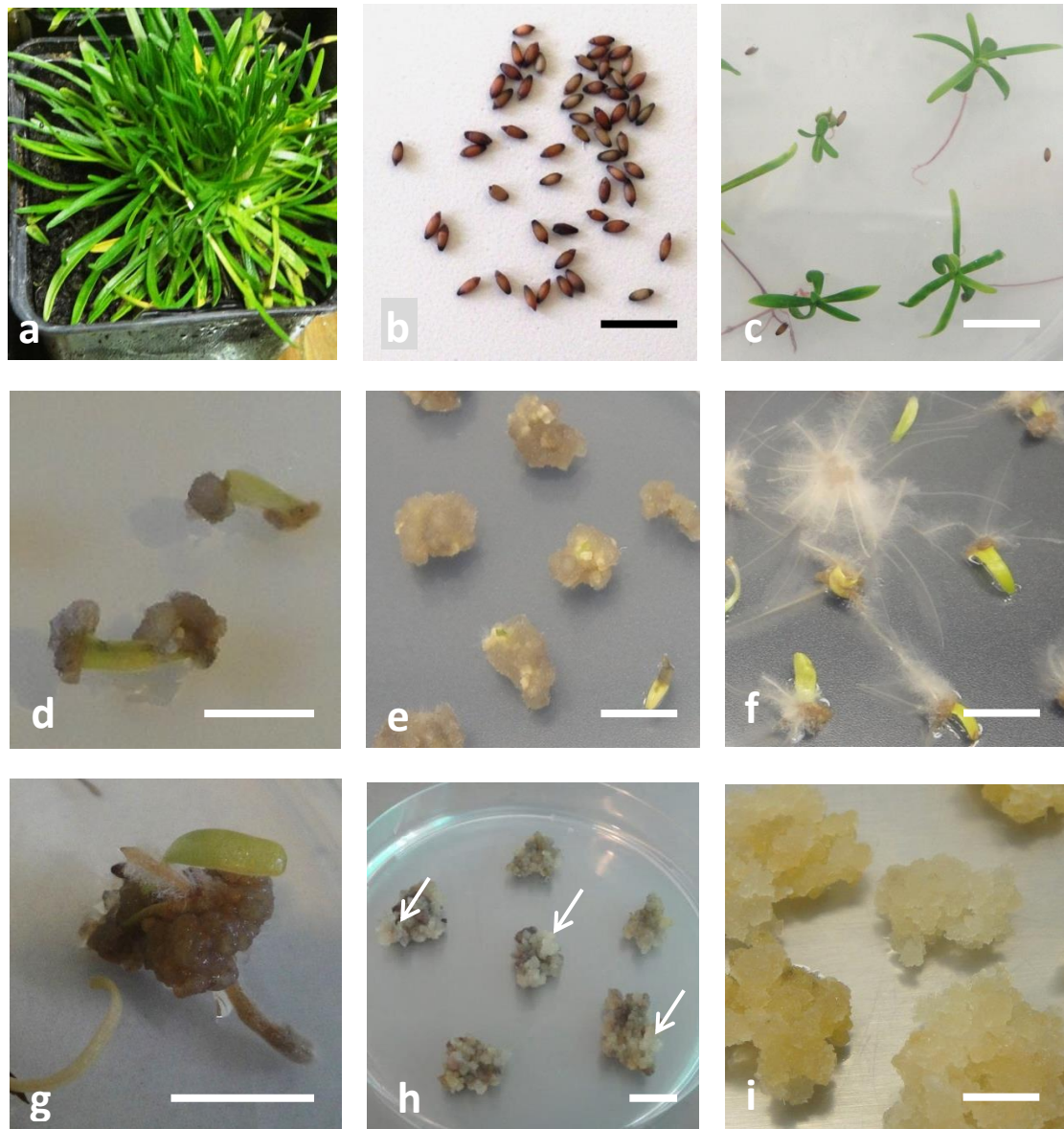


Fig. 1. Callus induction of *A. maritima*. (a) Plant cultivated in pots; (b) seeds ; (c) 6 weeks-old axenic seedlings; (d) 6 weeks and (e) 8 weeks leaf-derived calli on MS supplemented with 4.5 μM 2.4D, 0.93 μM KIN and 3% sucrose; (f) 8 weeks leaf-derived calli and rhizogenesis on MS supplemented with 2.7 μM NAA, 0.4 μM BA and 3% sucrose; (g) 8 weeks seed-derived callus on MS supplemented with 4.5 μM 2.4D, 0.93 μM KIN and 3% sucrose; (h) friable light yellow areas (arrows) developing on the brown primary seed-derived calli (i) multiplication of friable seed-derived calli. Bar =1 cm.

Table 1

Effects of different *Armeria maritima* explant types on callogenesis efficiency and callus quality.

Explant	Callus amount ¹ (% of explants forming calli)	Callus quality ²
Leaf from soil-grown plantlets	43.1 ± 18.5 ^a	brown, compact
Seed	20.8 ± 13 ^{ab}	pale yellow, friable
<i>Explants from axenic seedlings:</i>		
Cotyledon	0.8 ± 1.9 ^b	yellow, friable
Leaf	47.5 ± 28.8 ^a	pale brown, heterogeneous
Root	43.3 ± 16.1 ^a	brown, heterogeneous

¹Data ± SD collected after 8 weeks of culture in MS medium supplemented with 4.5 µM 2.4D, 0.93 µM KIN and 3% sucrose (pH 5.8). Means are expressed for % response from at least three experiments (n=40). Values sharing same letters are not significantly different according to Dunn test (p<0,1).

²Observed after subcultures of primary calli

Table 2

Effects of different growth regulators combinations on callus induction and rhizogenesis from several explants of *Armeria maritima*

Growth regulators (μM)				Explant forming callus (%)		
2,4-D	NAA	KIN	BA	Seed	Root ¹	Leaf ¹
0	0	0	0	$0.8 \pm 1.4^{\text{ab}}$	$0.8 \pm 1.4^{\text{ab}}$	0^{a}
4.5	0	0.93	0	$18.3 \pm 8.8^{\text{abc}}$	$43.3 \pm 16.1^{\text{c}}$	$47.5 \pm 28.8^{\text{c}}$
0	2.7	0	0.4	$27.5 \pm 6.6^{\text{bc}}$ + R	$37.5 \pm 32.7^{\text{c}}$ + R	$50.8 \pm 13.8^{\text{c}}$ + R
0	5.4	0	0.4	$20.8 \pm 8.0^{\text{abc}}$ + R	$45 \pm 18.9^{\text{c}}$ + R	$51.7 \pm 29.8^{\text{c}}$ + R

¹: Derived from 6 weeks old axenic seedlings.

+R: Numerous roots developed on calli.

Data \pm SD collected after 8 weeks of culture in MS medium supplemented with 3% sucrose (pH 5.8). Means are expressed as % response from six experiments (n=40). Values sharing same letters are not significantly different according to Dunn test ($p < 0,05$).

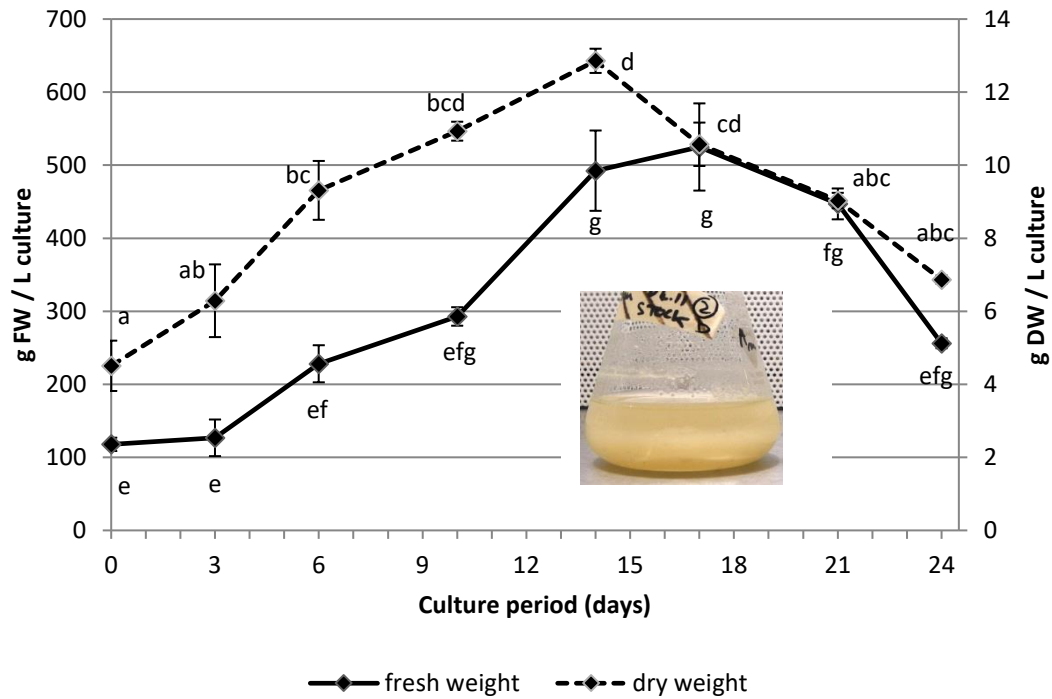


Fig. 2. Growth of cell suspension cultures of *A. maritima* in liquid MS medium supplemented with 4.5 μM 2.4D and 0.93 μM KIN during 24 days of culture at 20°C in the dark. FW– Fresh weight, DW – Dry weight. The bars indicate the standard error ($\pm\text{SE}$) from at least three independent measurements. Data annotated with same letters are not significantly different, according to Dunn test ($p < 0.05$). Inset picture shows a cell suspension culture of *A. maritima* at the end of its stationary phase (day 14).