In vitro morphogenetic response of *sarcocornia ambigua*

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ABSTRACT

Sarcocornia ambigua (Michx.) is a halophyte, which has a salt enriched with various minerals and low sodium content. This vegetable has pharmacological properties, of interest for the pharmaceutical and nutraceutical industry; however, there is no knowledge about the micropropagation of this vegetable. The present work evaluated the morphogenetic response of different explants in the *in vitro* culture of this species. The explants used were young and adult nodal segments, divided into apical, median and basal regions. The aseptic cultures were obtained from pre-disinfestation in 0.008% kasugamycin with neutral detergent and asepsis with 70% ethanol, 1% NaClO, 4% CaClO and 0.3% $HgCl_2$. The seed multiplication was performed in semi-solid or liquid MS medium, added with growth regulators 0.5 mg / L NAA and 1.0 mg / L BAP, containing or not NaCl and natural sediment from the marsh environment. The highest percentage of aseptic cultures of *Sarcocornia ambigua* was obtained from the explants of apical and basal nodal segments with pre-disinfestation in Kasugamycin and disinfestation with the use of 70% ethanol and 1% CaClO. The concentration of 20 g/L NaCl in the culture medium resulted in the best *in vitro* morphogenetic response of the explants. The natural marsh substrate present in the culture medium provided maintenance of the green explants longer. *Sarcocornia ambigua* showed good morphogenetic response capacity *in vitro*, depending on the age and position of the branch that provided the explant and the composition of the culture medium.

Index terms: *Halophyte;* Vegetative propagation; Micropropagation.

INTRODUCTION

Sarcocornia plants are halophytes, that is, tolerant to high saline concentrations, lethal to most other species (Alonso et al., 2008; Costa et al., 2014, Flowers; Colmer, 2008), among other strong characteristics related to their habitat as low availability of organic matter, direct and intense sunlight, flooding with saline water, etc. (Boeger; Gluzezak, 2006). Based on these peculiar characteristics, this species has been gaining prominence in several aspects.

As an alternative to the conservation of the marsh environment, the production of *Sarcocornia* seedlings (Ventura and Sagi, 2013), from the micropropagation, allows the recovery of saline areas (Muscolo, Panuccio and Piernik, 2014), performs the function as phytostabilizers of metals in polluted soils (Idaszkin et al., 2017) and as biofilters in different applications and cultures for saline agriculture (Buhmann et al., 2015, Glenn et al., 2013, Rozema and Schat, 2013, Shpigel et al., 2013; Webb et al., 2012). The last one as a possible alternative to increasing lack of fresh water (Hasanuzzaman et al., 2014).

Great interest is arising in the study of these halophytes due to their potential as a functional food, because of the presence of several nutrients and bioactive compounds (Buhmann; Papenbrock, 2013; Jang et al., 2007; Jeong et al., 2004; Ventura et al., 2011). Among these bioactive compounds with therapeutic properties found in these species, phenolic compounds deserve special emphasis. They have been object of studies due to their potential antioxidant properties, which result in different biological activities such as: anti-inflammatory, antitumor and antithrombotic activity (Barreira et al., 2017; Bertin et al., 2014; Isca et al., 2014; Lee et al., 2007; Pinheiro et al., 2017), being a potential product in the food and pharmaceutical industries, when cultivated under highly stressful conditions (Bertin et al., 2014; Flowers and Colmer, 2015).

The products generated from these organic compounds have been patented for pharmaceutical purposes in the treatment of tuberculosis (Rathod et al., 2006) and hypertension (GHOSH et al., 2007) This last illness is responsible for reaching 32.5% (36 million) adults, over 60% of the elderly, contributing directly or indirectly to 50% of cardiovascular disease deaths (Scala, Magalhães and Machado, 2015).

With other *Sarcocornia* species that occur in Europe and Asia, *S. ambigua* could be used as dietary and nutraceutical food in Brazil, provided there was a commercial culture of this species. The difficulty of the

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industry in obtaining a large quantity and quality of the vegetal material of this species derives from the difficulty that many producers have in producing their seedlings or acquiring them in the market.

The lack of information on *in vivo* vegetative propagation of *Sarcocornia ambigua* has been a major obstacle to obtaining this nutraceutical raw material. In order to succeed in the micropropagation of a species, it is necessary to define the ideal conditions for *in vitro* cultivation aiming the development of the plant since the *in vitro* morphogenetic response is dependent on the genotype and the culture conditions. The present work evaluated the effect of different sources of explants and culture conditions on the *in vitro* morphogenetic response of this species.

MATERIAL AND METHODS

Sterilization methods

The present study performed with plants of the species *Sarcocornia ambigua,* collected in the month of February (summer) of 2010, in the marsh environment of the Aririú river estuary – Palhoça/SC, Brazil. The work was carried out at the Vegetable Cell Cultivation Laboratory of the Earth and Sea Technology Center of the University of Vale do Itajaí.

The plant material for micropropagation consisted of young and adult nodal segments, which were approximately 2.5 mm in diameter, divided into apical, median and basal regions (Figure 1). The explants were removed from the plants collected (*Sarcocornia ambigua*) with the aid of a scalpel, and washed in running water, followed by immersion in distilled and sterilized water. For the pre-disinfestation, in laboratory bench, this material was immersed in 0.008% kasugamycin solution (Kasumin). For the asepsis performed in laminar flow chamber, the explants were submitted to 5 combinations of different chemical agents and immersion times: 1- Kasumin 20 h; Ethanol 70% 2 min; 1% CaClO 30 min (TA01 to TA06); 2- Kasumin 20 h; Ethanol 70% 2 min; 4% CaClO 10 min (TA07 to TA12); 3- Kasumin 20 h; Ethanol 70% 2 min; 0.3% $HgCl_2$ 4 min (TA13 to TA18); 4- Kasumin 15 h; Ethanol 70% 1 min 30 sec; 1% NaClO 25 min (TA19 to TA20); 5- Kasumin 15 h; Ethanol 70% 1 min 30 sec; CaClO 4% 8 min (TA21 to TA22).

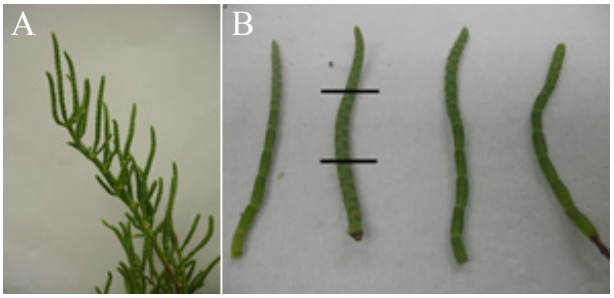

Figure 1 – *Sarcocornia ambigua* plant material used for the establishment of aseptic cultures. **A** - Branch containing nodal segments; **B** - Sectional nodal segments in apical (a), median (m) and basal (b) regions. (Photo: Janaina Rubio Gonçalves).

At the end of the asepsis, the explants were submitted to three washes with sterilized distilled water. They were then inoculated into 100 mL glass vials containing 15 mL of Murashige and Skoog (1962) semi-solid culture medium (MS), sterilized at 120 °C ± 1 °C and pressure of 1.3 kgf/cm² (1 atm) . Each treatment consisted of five replicates, containing one explant per plot (vial).

Culture medium for multiplication of plant material

The *Sarcocornia ambigua* multiplication was performed using young and adult branches, measuring 2.0 cm, divided into apical, median and basal regions, in semi-solid medium MS added with growth regulators 0.5 mg/L naphthalene acetic acid (NAA), and 6-benzylaminopurine (BAP) 1.0 mg/L with and without NaCl (10 20 and 30 g/L) and liquid MS containing sediment removed from the natural habitat of the parent plants. The natural sediment (NS) containing organic matter, small sticks, and shells; and the natural selected sediment (NSS) that went through the process of washing in running water, removing the sticks and shells. Both SN and SNS sediments were autoclaved and 35 grams of these were added to the liquid MS medium. The pH was adjusted to 5.75 ± 0.01 before autoclaving. The cultures were incubated in a growth room at 25 ± 2 °C, the relative humidity of the air, approximately 60%, fluorescent cold white light, an intensity of 50 µmol m^{-2} s^{-1} and photoperiod of 16/8 hours of light/dark.

The experiments were carried out in a completely randomized experimental design with five replicates per treatment.

The percentage data obtained in the assays were transformed into arccosine square root of x ($\sqrt{x}$), and

then, subjected to analysis of variance and the means were supported by the Tukey test at a significance level of 5%, by the VARPC program.

RESULTS AND DISCUSSION

Disinfestation of explants

In this study, the use of different combinations of chemical agents and immersion times promoted high rates of contamination, oxidation, and depigmentation, but also reached high survival rates (Table 1). Pre-disinfestation with 0.008% kasugamycin followed by asepsis treatments resulted in contamination values between 0 and 80%, oxidation up to 40%, depigmentation up to 60%, and survival up to 100% during 30 days. Although this fungicide is a viable alternative for the control of pathogens, the concomitant use of other disinfestation agents, sodium hypochlorite (1% NaClO), calcium hypochlorite (CaClO 4%) and mercury chloride ($HgCl_2$ 0.3%), as well as the culture medium helps to

Table 1 – Percentage of contamination, oxidation, depigmentation and survival of *Sarcocornia ambigua* explants submitted to different chemical agents and cultured in semi-solid Murashige and Skoog (1962) (MS) medium after 30 days of *in vitro* culture (N=5).

Treatment Code	Nodal Segment Type	Aseptic Treatment	Fungus Contamination (%)	Oxidation (%)	Depigmen-tation (%)	Survival (%)
TA01a	AYNS	ATa Kasumyn 20 h; Ethanol 70 % 2 min; NaClO 1% 30 min	0 a	0 a	0 a	100 a
TA02a	AANS		60 a	0 a	0 a	40 ab
TA03a	YMNS		0 a	0 a	0 a	100 a
TA04a	AMNS		60 a	0 a	0 a	40 ab
TA05a	YBNS		20 a	0 a	0 a	80 ab
TA06a	ABNS		60 a	0 a	0 a	40 ab
TA07b	AYNS	ATb Kasumyn 20 h; Ethanol 70 % 2 min; CaClO 4% 10 min	0 a	20 a	40 a	40 ab
TA08b	AANS		60 a	0 a	0 a	40 ab
TA09b	YMNS		0 a	0 a	0 a	100 a
TA10b	AMNS		80 a	0 a	0 a	20 b
TA11b	YBNS		40 a	0 a	0 a	60 ab
TA12b	ABNS		40 a	0 a	20 a	40 ab
TA13c	AYNS	ATc Kasumyn 20 h; Ethanol 70 % 2 min; $HgCl_2$ 0,3% 4 min	0 a	40 a	0 a	60 ab
TA14c	AANS		40 a	0 a	20 a	40 ab
TA15c	YMNS		0 a	40 a	60 a	0 b
TA16c	AMNS		20 a	0 a	0 a	80 ab
TA17c	YBNS		0 a	40 a	20 a	40 ab
TA18c	ABNS		40 a	0 a	20 a	40 ab
TA19d	AYNS	ATd Kasumyn 15 h; Ethanol 70 % 1 min 30 seg; NaClO 1% 25 min	20 a	0 a	0 a	80 ab
TA20d	YBNS		20 a	0 a	0 a	80 ab
TA21e	AYNS	ATe Kasumyn 15 h; Ethanol 70 % 1 min 30 seg; CaClO 4% 8 min	40 a	0	0 a	60 ab
TA22e	YBNS		80 a	0	0 a	20 b

* AYNS = Apical Young Nodal Segment; AANS = Adult Apical Nodal Segment; YMNS = Young Median Nodal Segment; AMNS = Adult Median Nodal Segment; YBNS = Young Basal Nodal Segment; ABNS = Adult Basal Nodal Segment.

control contamination in micropropagation. However, as the concentration increases and the immersion time in NaClO, CaClO and $HgCl_2$ promoted oxidation, depigmentation or even necrosis of the plant tissues, that was also observed by Jafari, Daneshvar and Lotfi-Jalalabadi, (2016); Moradpour, Aziz and Abdullah (2016).

Although the use of 4% CaClO and 0.3% $HgCl_2$ showed high rates of oxidation and depigmentation, relevant survival rates were also obtained. On the other hand, the use of 1% NaClO in this study, regardless of the immersion time, did not provoke oxidation and depigmentation and promoted high levels of *Sarcocornia ambigua* explant survival. However, despite the survival observed in the TAc, the use of mercury chloride ($HgCl_2$) was the treatment that most provoked oxidation and depigmentation, leading to the death of the explants, probably due to their phytotoxicity.

Asepsis treatments promoted a higher rate of aseptic cultures TA01a and TA19d for AYNS (100 and 80%, respectively); TA03a and TA09b for YMNS (100%); TA16c for AMNS (80%) and TA05 for YBNS (80%). The explants from the median region allowed obtaining a higher percentage of aseptic cultures when compared to the apical and basal ones. However, the apical and basal nodal segments presented better morphogenetic response *in vitro*, that is, they remained with dark green color and showed development of shoots (Table 2).

The young nodal segments, mainly in the apical and medial regions, presented the best survival rates, despite the shorter time of exposure to chemical agents. According to Sandhu, Wani and Jiménez (2018), Stevens and Pijut (2017), factors such as age and regions of the explants may also be determinants for the *in vitro* establishment of plant material.

The results of the present study corroborate with several other authors who observed rapid *in vitro* morphogenetic responses and high sprout rates in apical explants (Navarro-García, Morte; Pérez-Tornero, 2016; Prakash, Ramachandra; Hanur, 2015; Satish et al., 2015; Tallón et al., 2013). These regions become responsive due to the presence of actively divided cells in the meristematic zones of the explants (Çeliktas et al., 2006; Karatas et al., 2013). However, explants from the basal region also showed high levels of seed development. In a study conducted by Singh et al. (2015) with *Salicornia brachiata* callus formation was observed when nodal segments were placed vertically in the middle, indicating the presence of competent organogenic cells in the basal part of the explants.

Multiplication phase

Cultivation of the different types of *Sarcocornia ambigua "in vitro"* explants resulted in a varied response to development and survival (Table 2). The development of *in vitro* explants was reduced, but the segments of the apical, medial and basal regions presented shoots of 40, 20 and 60%, respectively, in the medium MS + 0.5 mg/L NAA + 1.0 mg/L BAP. The young apical and basal nodal segments had higher rates of depigmentation, but a lower rate of fungus contamination and a higher rate of sprouting explants. However, there was no survival of the explants regardless of the type and region of the material used. Raposo and Morais (2014), using the same concentration of BAP (1mg/L) as the present study, affirm that they do not induce the multiplication of shoots at this concentration in *Sarcocornia fruticosa (L.) A. J. Scott.*

On the other hand, when *S. ambigua* explants were cultivated in the same culture medium added with 10, 20 and 30 g/L NaCl, the sprouting rate increased from 40 to 100%, except for TM12d treatment for YBNS, which did not occur sprouting. This result corroborates with other studies that showed better morphogenetic responses when added NaCl in halophyte culture media, such as an increase in respiratory rates, number of seeds and flowers, vegetative biomass and photosynthetic efficiency (Alonso et al., 2017; Asrar et al., 2017; Guo et al., 2018; He, Silliman; Cui, 2017; Redondo-Gómez et al ., 2006; Singh et al., 2015). However, the presence of salt above a specific concentration limit for each species can be lethal to the plant.

When using the MS medium + 0.5 mg/L ANA + 1.0 mg/L BAP, but added with natural sediment, the shoot formation was 20% for AYNS and YBNS and zero percent when the selected natural sediment was used. The YBNS segments presented the best sprouting rates per explant, but only the AYNS obtained survival rates ranging from 20% to 60%, while YBNS presented zero percent of survival in all treatments.

Table 2 – Percentage of sprouting, survival and oxidation of *Sarcocornia ambigua* species explants cultivated in Murashige and Skoog (1962) (MS) medium added with naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) after 106 days for TM01 to TM04 and 60 days for treatments TM05 to TM07.

Treatment Code	Nodal Segment Type	Treatment of Culture Medium (TM)	Sprouting Explants (%)	Oxidation (%)	Depig-mentation (%)	Survival (%)
TM01a	* AYNS	TMa MS + 0.5 mg/L ANA + 1.0 mg/L BAP	40 b	0 b	60 ab	0 b
TM02a	*AANS		0 b	0 b	20 b	0 b
TM03a	*YMNS		20 b	0 b	60 ab	0 b
TM04a	*AMNS		0 b	0 b	0 b	0 b
TM05a	*YBNS		40 b	0 b	60 ab	0 b
TM06a	*ABNS		20 b	0 b	40 b	0 b
TM07b	*AYNS	TMb MS + 0.5 mg/L ANA + 1.0 mg/L BAP + 10 g/L NaCl	40 b	0 b	60 ab	40 ab
TM08b	*YBNS		40 b	0 b	40 b	0 b
TM09c	*AYNS	TMc MS + 0.5 mg/L ANA + 1.0 mg/L BAP + 20 g/L NaCl	40 b	0 b	100 a	0 b
TM10c	*YBNS		100 a	0 b	100 a	0 b
TM11d	*AYNS	TMd MS + 0.5 mg/L ANA + 1.0 mg/L BAP + 30 g/L NaCl	40 b	0 b	80 ab	0 b
TM12d	*YBNS		0 b	0 b	40 b	0 b
TM13e	*AYNS	TMe MS + *SN + 0.5 mg/L ANA + 1.0 mg/L BAP	20 b	40 ab	0 b	60 a
TM14e	*YBNS		20 b	60 a	0 b	0 b
TM15f	*AYNS	TMf MS + *SNS + 0.5 mg/L ANA + 1.0 mg/L BAP	0 b	20 ab	40 b	20 ab
TM16f	*YBNS		0 b	40 ab	0 b	0 b
TM17g	*AYNS	TMg MS liquid + 0.5 mg/L ANA + 1.0 mg/L BAP + 20 g/L NaCl	100 a	0 b	80 ab	20 ab
TM18g	*YBNS		80 ab	0 b	100 a	0 b

*AYNS = Apical Young Nodal Segment; AANS = Adult Apical Nodal Segment; YMNS = Young Median Nodal Segment; AMNS = Adult Median Nodal Segment; YBNS = Young Basal Nodal Segment; ABNS = Adult Basal Nodal Segment; ** NS = Natural Substrate; SNS = Selected Natural Substrate.

Corroborating with the present study, in which the use of the natural substrate (NS) promoted the shoots while the natural substrate selected did not, Duan et al. (2007), observed that in single salt solutions there was greater inhibition of germination when compared to solutions extracted from the soil with multiple salts. However, regardless of the presence or absence of shoot formation, the substrate in the culture medium provided maintenance of the green explants for a longer period.

The process of removal of sticks and shells from the SNS and in the process of washing in running water may have caused the loss of some nutrient from the medium, including NaCl and other salts, necessary to induce sprouts. The culture media with the selected natural sediment provided a lower sprouting rate relative to the added NaCl media, despite high oxidation and survival rates of the explants. When these were grown in the liquid MS medium added with 20 g/L NaCl there was an increase in shoot rate reaching 100% and survival of 20%.

The use of the liquid MS medium in the treatments (TM17g and TM18g) provided a higher index of sprouting explants for both AYNS (100%) and YBNS (80%). Despite this favorable result, a high depigmentation rate of the explants occurred, resulting in only 20% survival for AYNS and 0% for YBNS. The damage caused by high levels of salinity in halophytes may also have been responsible

for high rates of depigmentation of explants in this study. however, when it comes to the micropropagation of Salicornias, Raposo and Morais (2014) state that these species depend more on other factors such as the number of explant nodes (1 to 3 nodes) than on nutritional supplements, although this factor has not influenced in the present study, once explants with 3 or more nodes (2 cm) were used. Factors such as the type of explants, medium, volume of the medium and repeated transfer time period significantly influence tissue growth in culture.

The establishment of aseptic crops by means of explants disinfestation methods is one of the most difficult stages of micropropagation. In the present study, the ambiguous Sarcocornia species presents a stem and foliar anatomy that hinders the elimination of contaminant agents. The surface of the explant nodal segments has a very irregular epidermis, wavy and with reentrances, which makes it difficult for the disinfecting agents used to contact and act on the contaminant microorganisms. Therefore, the explants were subjected to higher times and concentrations to the disinfecting agents, but these treatments promoted the oxidation and depigmentation of the explant, especially in the young nodal segments.

The consistency and composition of the culture medium also influenced the response of explants *in vitro*. The presence of 0.5 mg/L ANA; 1.0 mg/L BAP and 20 g/L NaCl in MS medium resulted in obtaining a higher budding rate, especially when the medium was liquid. Thus, it is evident that *in vitro* cultivation of ambiguous Sacocornia is possible, if care is taken in the treatments of explants asepsis and in the formulation of the culture medium, aiming to allow its morphogenesis.

CONCLUSIONS

It is possible to establish and maintain aseptic cultures of *Sarcocornia ambigua in vitro* from the explants of the nodal segments, in the liquid MS culture medium with the use of growth regulators, NaCl and natural substrate of the marsh environment.

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