Micropropagation of the Halophyte *Sarcocornia fruticosa* (L.) A. J. Scott

Maria Filomena de Jesus Raposo and Rui Manuel Santos Costa de Morais*

CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal

Abstract: Details of investigation to evaluate the effects of the number of nodes (one, two or three) of *Sarcocornia fruticosa* explants on growth and multiplication rate of plantlets are presented in this paper. The responses of the 3-node explants to some supplementary sources of different aminoacids and growth regulators indol-3-acetic acid, 6-benzylaminopurine and gibberellins A3 were also analysed. Plantlets from 3-node explants showed a marked increase in growth and number of lateral shoots, indicating that *Sarcocornia* does not respond well when explants are very small. The addition of 100 mg l^{-1} casein hydrolysate plus 150 mg l^{-1} glutamine, and 100 mg l^{-1} casein hydrolysate plus vitamins showed to be good growth promoters in micropropagating *Sarcocornia*, giving longer plantlets and higher multiplication rates.

Keywords: Explants, halophyte, *in vitro* propagation, marine biology, *Sarcocornia*.

INTRODUCTION

Currently, with the increase of soil salinity either because of natural causes or due to agricultural techniques used, finding adequate agricultural fields and water to irrigate them has become a challenge to be overcome. Hence, salt-tolerant and halophytic plants could be one solution. Besides, the potential of old abandoned salt-marshes could be used to advantage, the tides accounting for the irrigation. *Sarcocornia fruticosa* (Chenopodiaceae) is a perennial succulent halophyte plant with articulate stems and leaves reduced to a scale, which could be used for this cultivation purpose.

Salicornia is another genus whose morphology and biochemical composition are closely related to *Sarcocornia*, despite the annual character of *Salicornia.* Both genera are commonly known as salicornias.

Salicornias, in general, are very rich in iodine, phosphorus, calcium, silicon, zinc, manganese, and vitamins A, C and D [1], and also in diuretic, depurative and resolutive (curative) substances. Seeds are rich in edible oils (26-30% of the total lipids), highly unsaturated, linoleic acid contributing with 67% for the total fatty acid content, followed by oleic (17.5%) and linolenic (1.4%) acids. Salicornias, like other vegetables, could be a very good source of ω-6 (linoleic acid) and ω-3 (oleic acid) fatty acids that are essential for humans, since they cannot synthesize some of the fatty acids [2]. Besides, linoleic and linolenic acids, after suffering desaturation and elongation by some desaturases and elongases, can be converted into arachidonic acid (ARA), and eicosapentaenoic (EPA) and docosahexahenoic (DHA) acids, respectively. Moreover, ARA and EPA are natural precursors of prostaglandins, leukotrienes and hydroxy fatty acids, among other important compounds. ARA, for example, has been associated with prevention/treatment of some cardiovascular diseases, because of its pro-aggregative and vasoconstritive actions on the platelet, and antiaggregative and vasodilatative actions on the endothelium. On the other hand, EPA exhibits some effects as anti-thrombotic, anti-arrhythmic and anti-inflammatory agent, and DHA facilitates normal growth, development and function of the normal nervous system. Besides, both EPA and DHA lower lipid content [3] and reduce cholesterol and triglycerides in plasma [4].

Salicornia and *Sarcocornia* contain L-ascorbic acid that can act as an oxygen reducing agent or regenerating primary antioxidants, principally tocopherols, and also as a free radical scavenger [5].

In addition, major (calcium, zinc) or minor minerals (iodine) are essential for the building up of and maintenance of a good/healthy skeleton (bones and cartilage formation), immune and nervous systems, and to maintain the electrolyte imbalance of the body.

But growth of salicornias in the field depends on the seasons of the year and also on the production and germination of seeds. *In vitro* propagation can overcome these problems and can also provide a large

*Address correspondence to this author at the CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal; Tel: +351 22 5580050; Fax: +351 22 5090351; E-mail: rcmorais@porto.ucp.pt

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number of plants with the same phenotypic and genetic characteristics, free of pathogens, throughout the year.

Nevertheless, information on the *in vitro* propagation of salicornias is scarce, with most of the experiments and observations relating to field growth and ecology. Onaindia and Omezaga [6], for example, had already referred that *Salicornia ramosissima* (a related species of *Sarcocornia*) prefers locations rich in organic matter, and Prehn *et al.* [7] and Ahmad and Anis [8] verified that casein hydrolysate and glutamine provided good results in the micropropagation of *Quillaja* and *Cucumis*.

Other growth promoters have been used with several species. For example, Stefaniak *et al.* [9] used 6-benzylaminopurine (BAP) and indol-3-acetic acid (IAA) to obtain a quicker growth of *Salsola*. Mei *et al*. [10] also used the same growth regulators to break dormancy of *Atriplex*. Other groups [11-13] used other growth regulators, like indolbutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) or kinetin (kin), with different plants, such as *Avicennia*, *Triticum*, *Vigna sinensis,* and *Zea mays*.

The aims of this work were to test the hypothesis that casein hydrolysate and glutamine are good growth promoters for *Sarcocornia fruticosa,* and to evaluate how the number of nodes of the explants affects the micropropagation and multiplication rates.

MATERIALS AND METHODS

Biological Material and First Culture Conditions

Seeds and explants of *Sarcocornia fruticosa* were used to carry out this piece of work. Seeds were collected in November 2005, in the wetland and salt marshes of Algarve, the southern part of Portugal, and were stored in a dark place, under room temperature (20-25 ºC), until the end of the project (during one year).Two sets of experiments were conducted to analyse the best conditions of plant growth and micropropagation.

Culture medium for germination of seeds was H&A [14], supplemented with 2 % NaCl (w/v), and 1 % agar (w/v). Medium pH was corrected to 7.15-7.20, as referred to by Davy *et al.* [15].

For culturing and *in vitro* propagation, culture medium was initially the same but, as the experiments progressed, concentrations of nutrients, especially nitrogen and phosphorus, proved to be insufficient for growth and development of plantlets. Since then, the medium was used in a double strength factor (2H&A).

Each experiment consisted of six replicates and was carried out carried out in a *walk-in* chamber, with constant 24 hour-period light (29 $\mu E\ s^{-1}\ m^{-2}$) and temperature (25 ºC), in order to accelerate the growth. Each replicate consisted of one explant or plantlet.

Practical work began with the disinfection of seeds that were put to germinate. Then, as soon as plantlets were high enough and presented already several shoots, explants with one, two and three nodes were obtained and subjected to different treatments as shown below (Number of nodes and enrichment of the growth medium). Explants with three nodes were used thereafter in other experiments.

Seed Disinfection and Germination

Seeds were surface sterilised following these steps: ethyl alcohol 70 % (v/v), 5 min; sterile deionised water, 1 time; sodium hypochlorite 2.5 % (v/v) + 0.5 % tween 20 (v/v), 10 min; sterile deionised water, 3 times; and Benomil 1 % (w/v), 2 min.

Seed germination was carried out in Petri dishes with 30 ml solidified H&A medium.

Number of Nodes and Enrichment of the Growth Medium

Since using too small explants (with less than 5 mm) did not grow and tissues died, explants of the articulate stems, with 1, 2 and 3 nodes, were used in order to observe the influence of the explant length, and were subjected to different conditions: vitamins (Vit) + casein hydrolysate (CNH), 100 mg L^{-1}; CNH, 100 mg L^{-1}; Vit; control (without any enrichments). Vitamins were those from the B5 medium [16]. Eight weeks later plantlets were removed to simple 2H&A medium. Plantlets without roots were prior sowed in a 98 μM IBA solution, to induce rooting, before being transferred to the 2H&A simple medium.

Influence of BAP and IAA (Growth Regulators) and Enrichment of the Growth Medium

To induce growth of new shoots BAP (0.5 and 1.0 mg L^{-1}) was used according to Prehn *et al.* [7], and Ahmad and Anis [8], but other substances were added to enrich the medium, as given below:

2H&A (control, without any enrichments);

2H&A + CNH, 100 mg l^{-1};

2H&A + glutamine (gln), 150 mg L^{-1};

2H&A + CNH, 100 mg L^{-1} + gln, 150 mg L^{-1};

2H&A + CNH, 100 mg L^{-1} + gln, 150 mg L^{-1}+ BAP, 1 mg L^{-1};

2H&A + CNH, 100 mg L^{-1} + gln, 150 mg L^{-1} + BAP, 0.5 mg L^{-1};

2H&A + BAP, 1 mg L^{-1};

2H&A + BAP, 0.5 mg L^{-1};

2H&A + BAP, 0.5 mg L^{-1} + IAA, 0.1 mg L^{-1} (as in [10])

Influence of GA3 on the Development of Explants

To evaluate the difference gibberellic acid (GA_3) induces on the growth of the explants and on the development of new branches/shoots, we used this gibberellin (5 mg L^{-1}) with and without glutamine (gln, 150 mg L^{-1}) and/or casein hydrolysate (CNH, 100 mg L^{-1}), as given below:

C (control, 2H&A without any enrichments);

C+GA3;

C+GA3+gln;

C+GA3+CNH;

C+GA3+gln+CNH;

C+gln;

C+CNH;

C+CNH+gln.

Statistical Analysis

Statistical analysis was performed using Statistica 6.0 (StatSoft Inc.). Differences between means (One-way Anova) were assessed by the post-hoc Tukey's Honest Significant Difference (HSD) test. Values were considered significantly different at $p<0.05$.

RESULTS AND DISCUSSION

Sarcocornia did not seem to react positively to culturing of explants on media enriched with growth

Table 1: Length and Number of Nodes of the Main Shoot of *Sarcocornia* Plantlets, Number of Lateral Shoots and Number of Nodes/Shoot of 1 to 3-Node Explants of *Sarcocornia* Under Different Growth Media (During Seven Weeks), Seven Weeks After Being Transferred to a Medium with no Treatment

Growth medium	Nº of nodes of explants	Main axis/shoot		Lateral shoots	
		Length (mm)	Nº of nodes	Nº shoots	Nº of nodes/ shoot
2H&A	3	38±3 b	7.0±1.0	5.0±0.25	1
	2	34±3 bc	6.0*	4.0±0.3	1
	1	9±1 e	2.7±0.6	0	0
2H&A+CNH	3	38±5 b	6.7±0.6	1.0±0.3 2.0±0.25	2 1
	2	29±2 c	7.0*	1.0±0.25	1
	1	12±1 e	4.0±1.0	0	0
2H&A+Vit	3	29±3 bc	4.7±0.6	2.0±0.25	1
	2	37±5 b	7.0±1.0	1.0* 2.0±1.0	3 1
	1	18±1 d	4.8±0.5	0	0
2H&A+CNH+Vit	3	50±5 a	9.0*	4.0±0.3 4.0±0.25	4 1
	2	32±5 b	7.8±0.5	4.0* 2.0±0.3	3 1
	1	37±3 b	7.0±1.0	5.0±0.25	1

2H&A – simple medium (control); CNH – 100 mg L^{-1} casein hydrolysate; Vit – vitamins.
Same letter means no significant difference (Tukey test, $P<0.05$). Letters are listed only for relevant parameters.
*standard deviation is 0.

promoters/phytohormones as other plants. As a matter of fact, all the additives seemed to cause death of the fragile small explants (results not shown). After that, we started using explants with one to three nodes.

Number of Nodes and Enrichment of the Growth Medium

When comparing growth of the different *Sarcocornia* explants with 1 to 3 nodes after a 3-week period, it was already possible to observe a better development of explants with 3 nodes, under simple medium 2H&A, with no supplements. Moreover, more than the addition of casein hydrolysate (CNH) or vitamins (vit), the number of nodes of the explant was the main factor for the better growth of sarcocornias. One third of those 3-node explants had already showed out the principal root (results not shown).

After seven weeks in a medium enriched with 100mg L^{-1} CNH+Vit difference between growth of explants with different number of nodes was significant, those with 3 nodes presenting the best development and having a good rooting system (results not shown).

However, when vitamins were added alone, explants with 2 nodes apparently showed the best growth. However, both groups of 2- and 3-node explants presented one third of rooted plantlets (Table **4**). On the other hand, CNH alone did not induce differences on the growth of any of the explants (results not shown).

Seven weeks after being transferred to simple 2H&A medium, plantlets whose explants had been under CNH+Vit, grew further and already presented 4 shoots per explant, each with 3 nodes (Table **1**).

In general, treatment of 3-node explants with CNH+Vit was the most efficient, giving rise to better developed plants and higher multiplication rates, with more secondary shoots and more nodes per shoot. This effect was even more evident with plants coming from 1-node explants, which showed the best characteristics among similar explants under different development conditions (Table **1**).

Influence of BAP and IAA (Growth Regulators) and Enrichment of the Growth Medium

Development of the 3-node explants of *Sarcocornia* was affected by the compounds added to enrich the growth medium. Growth increased mainly with CNH+gln, presenting 75% rooted plantlets. These plantlets also showed more ramified secondary/lateral shoots (Table **2**), showing, therefore, higher multiplication rates. However, when growth medium was enriched with CNH+gln+BAP, it did not induce positive differences, in relation to the results obtained with CNH+gln (Table **2**). These results are in agreement with the ones of the first experiment. In both experiments, a significant increase in plant growth was verified when medium was enriched with casein hydrolysate (CNH) and vitamins (Table **1**) or glutamine (Table **2**). Similar results have already been recorded by Prehn *et al.* [7] with *Quillaja saponaria*. They also observed an increase of the main shoot length and rooting percentage, when 100 mg L^{-1} CNH and 150 mg L^{-1} glutamine (gln) were added to the growth medium. On the other hand, they obtained a higher number of secondary shoots with 1.0 mg L^{-1} BAP, whilst in our work with *Sarcocornia* this growth regulator did not induce shoot multiplication at this concentration. However, because Ahmad & Anis [8] had referred that concentrations of BAP higher than 2 μM (ca. 0.5 mg L^{-1}) led to the formation of *calli* in *Cucumis sativus* explants, we also worked with 0.5 mg L^{-1} (2.22 μM) BAP. We obtained a higher number of secondary/lateral shoots with 0.5 mg L^{-1} BAP+CNH+gln, than with 1.0 mg L^{-1} BAP+CNH+gln. Nevertheless, the highest multiplication rate was obtained with the 3-node explants, under CNH+Vit, with an average of nine lateral shoots, four with four nodes and four with one node (Table **1**).

This assay also showed that micropropagation of salicornias depends more on the number of nodes of the explants than on nutritional supplements. For a good development we need at least two-node explants, growth always being better when 3-node explants are used.

Influence of GA3 on the Development of Explants

Gibberellins are involved in the regulation of cell elongation; they determine plant height and fructification and thus they are economically important [17].

Several workgroups also observed that application of gibberelic acid (GA3) could improve shoot growth of *Xanthium pennsylvanium* [18], *Quercus robur* [19], and *Prunus avium* [20]. Nevertheless, *Sarcocornia* did not answer positively to the GA3 enrichment of the medium. In fact, after 6 weeks of treatment with GA3 (Table **3**), all explants presented a growth depression when compared with control plantlets, and growth

Table 2: Length and Number of Nodes of the Main Shoot of *Sarcocornia* After a 4-Week Period of Different Treatment Conditions, and Results of 4 and 6 Weeks After Transferring Plantlets to the Simple 2H&A Medium. Number of Lateral Shoots and Number of Nodes Per Shoot are also Indicated

	After 4 weeks of treatment		After 4 weeks 2H&A medium				2 weeks later			
	Main axis/shoot		Main axis/shoot		Lateral shoots		Main axis/shoot		Lateral shoots	
	Length (mm)	Nº of nodes	Length (mm)	Nº of nodes	Nº of shoots	Nº of nodes/ shoot*	Length (mm)	Nº of nodes	Nº of shoots	Nº of nodes/ shoot*
2H&A (control)	8.5±1.7	2.8±0.5	10.0±2.0 e	3.3±0.58	0.3±0.06	1	-	-	-	-
CNH	17.0±1.7	5.3±1.15	28.0±5.3 ab	5.7±1.53	1.7±0.58 1.7±0.58	2 1	44.0±4.0 b	7.5±0.71	1* 3±1 1*	3 2 1
Gln	12.0±1.0	3.7±0.5	12.3±0.6 e	4.0±1.0	0.3±0.58 0.3±0.58	2 1	35.0±5.0 b	6.3±2.52	0.3± 0.06 1.7± 0.58	3 2
CNH+gln	19.0±9.1	4.8±0.96	33.3±6.5 a	7.0±1.73	1.0* 1.8±0.5	2 1	65.0±7.1 a	9.0*	3* 2.7± 0.58	2 1
CNH+gln+0.5BAP	16.0±2.6	4.0±1.0	21.3±2.9 b	4.7±0.58	1.3±0.58 1.0*	2 1	22.0±3.9 c	4.7±0.58	1.7± 0.58 1*	2 1
CNH+gln+1BAP	17.3±1.2	4.3±1.15	17.3±1.2 cd	5.0±1.0	1.0* 1.0*	2 1	23.0±3.2 c	5.0±1.0	1* 1.3± 0.96	2 1
0.5BAP	9.3±1.5	3.3±0.5	17.3±5.5 bcde	5.7±1.53	0.7±1.15	1	25.0±7.2 bc	5.3±1.15	0.3± 0.58	2
1BAP	12.2±1.1	5.0±1.0	13.6±1.5 de	4.0*	0.5±0.58	1	-	-	-	-
0.5BAP+0.1IAA	11.5±1.7	4.0*	14.5±1.0 de	4.5±0.58	0.3±0.58	1	17.5±5.0 c	5.0*	0.5± 0.58	1

2H&A – simple medium (control); CNH – 100 mg L^{-1} casein hydrolysate; gln –150 mg L^{-1} glutamine; BAP – 6-benzylaminopurine; IAA – indolacetic acid.
Values with the same letter are not significantly different (Tukey test, P<0.05). Letters are listed only for relevant parameters.
*standard deviation is 0.

Table 3: Length and Number of Nodes of the Main Shoot of *Sarcocornia* After a 6-Week Period of Different Treatment Conditions, and Results of 3 and 6 Weeks After Transferring Plantlets to the Simple 2H&A Medium. Number of Lateral Shoots and Number of Nodes Per Shoot are also Indicated

	After 6 weeks of treatment		After 3 weeks 2H&A medium				6 weeks later			
	Main axis/shoot		Main axis/shoot		Lateral shoots		Main axis/shoot		Lateral shoots	
	Length (mm)	Nº of nodes	Length (mm)	Nº of nodes	Nº of shoots	Nº of nodes/ shoot	Length (mm)	Nº of nodes	Nº of shoots	Nº of nodes/ shoot
C (2H&A)	31.3±0.82 a	4.7±0.52	38.5±1.29 b	5.5±1.05	4.0*	2.0*	77.3±1.50 b	10.2±0.75	12.5±1.0	3.5±0.55
C+GA3	19.0±0.82 c	3.4±0.55	23.5±1.29 c	4.5±0.58	2.3±1.03	1.9±0.55	59.3±2.63 c	7.8±2.22	7.0±1.41	3.0±0.71
C+GA3+gln	19.3±0.96 c	3.3±0.96	20.5±1.73 c	3.5±0.58	2.0*	1.8±0.96	44.0±2.65 d	7.0±1.73	3.7±2.08	4.0±1.0
C+GA3+CNH	15.8±0.96 d	3.0*	20.5±2.08 c	3.8±1.26	4.3±1.71	2.4±0.48	75.7±4.04 b	9.3±1.53	7.3±3.51	3.2±0.45
C+GA3+gln+CNH	16.3±1.26 d	3.3±0.5	20.5±2.08 c	4.3±0.96	3.3±0.96	3.0*	72.0±2.65 b	10.7±1.15	11**±1.41	5.0±0.82
C+gln	21.0±1.41 c	4.0*	37.5±1.71 b	6.2±0.75	3.3±1.26	1.0*	74.3±4.51 b	10.3±1.15	12±2.00	2.8±0.45
C+CNH	26.5±1.91 b	4.3±0.96	37.3±1.89 b	6.3±0.5	3.3±1.50	2.3±0.50	73.3±2.63 b	10.7±0.82	9.0±2.45	3.2±0.41
C+CNH+gln	32.8±1.50 a	5.0*	55.0±2.16 a	6.4±0.55	6.0*	1.6±0.55	88.5±1.29 a	10.8±0.84	11.2±0.84	2.8±0.45

2H&A – simple medium (control); CNH – 100 mg L^{-1} casein hydrolysate; gln –150 mg L^{-1} glutamine.
Values with the same letter are not significantly different (Tukey test, P<0.05). Letters are listed only for relevant parameters.
*standard deviation is 0.
** some lateral shoots already ramified.

depression was even more evident nine weeks after transferring plantlets to the simple 2H&A medium. Plants that had been treated with GA3 alone (C+GA3) or in combination with glutamine (C+GA3+gln) were the smallest, the latter ones showing half of the height of the "C+CNH+gln" treated plants.

Moreover, in contrast with the results observed by Anand *et al.* [21]) and Ford *et al.* [20] who obtained an improvement of rooting either with *Ipomoea* and *Prunus* cuttings, development of salicornias roots was severely inhibited when treated with GA3+CNH (Table **4**). All the plants previously treated with GA3 showed a root growth depression, as observed by other authors on different kinds of plant cuttings [19, 22-24]. One of the explanations for rooting inhibition could be that explants were exposed to light as it happened with Carvalho *et al.* [22] and Carvalho [25].

Table 4: Effects of Gibberellic Acid (GA3), Glutamine (gln) and Casein Hydrolysate (CNH) on the Rooting of *Sarcocornia*

Enrichment of the growth medium	Number of rooted plants (%)
Control (2H&A simple medium)	100
C+GA3	50
C+GA3+gln	67
C+GA3+CNH	33
C+GA3+gln+CNH	67
C+gln	100
C+CNH	100
C+gln+CNH	100

During this assay, best growth results were obtained when explants were treated with a medium enriched with casein hydrolysate (CNH) plus glutamine (gln) (Table **3**). Nine weeks after being treated and transferred to simple medium, these plants showed a growth significantly higher than the plants from all the other conditions. Moreover, by the end of treatment (first 6-week period), all of them were already rooted (Table **4**).

In addition, "CNH+gln" induced a decrease in the differences between plants of the same group, plants obtained being more homogeneous and regular despite the reduced number in nodes/shoot of the lateral shoots. Actually, this combination promoted lateral shooting when GA3 was also added to the medium (Table **3**).

Data obtained from these experiments indicate that supplementary sources of nitrogen, in the form of aminoacids (glutamine) and oligopeptides (casein hydrolysate), appear to be necessary for micropropagation of salicornias, along with explants with, at least, three nodes.

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