تأثير بعض السكريات في تجديد نبات *Obione portulacoides* (L.) Moq. مخبرياً دون استخدام إضافات هرمونية

Influence of some Sugars on *In Vitro* Micropropagation of *Obione portulacoides* (L.) moq. Explants on Hormone-free Media

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الملخّص

تم بإستخدام تقانة الزراعة في الأنابيب إكثار لبراعم نبات *Obione portulacoides* (L.) moq وهي شجيرة صغيرة من النباتات الملحية تنتشر بشكل واسع في أوراسيا المعتدلة وأجزاء من افريقيا، تنمو في المستنقعات المالحة والكثبان الساحلية، تمت دراسة تأثير إضافة بعض السكريات مثل السكروز والفركتوز والكزايللوز في مختلف أشكال استجابة النبات الشكلية في ظل غياب منظمات النمو. أمكن في هذا البحث الحصول على نباتات كاملة دون إضافات هرمونية، حيث سُجل أعلى متوسط للبراعم (4.2) والجذور (6.3) المتشكلة عند إضافة خليط الفركتوز و الكزايللوز إلى وسط الزراعة، وقد كان أفضل نمو للساق 4.3 سم عند إضافة خليط الفركتوز و الكزايللوز إلى وسط الزراعة، تلاه 2.9 و 4.2 على التوالي بوجود الكزايللوز فالسكروز. جذرت النباتات بشكل أفضل بوجود الكزايللوز في وسط الزراعة (9.3 مغ وزن جاف/نبات)، ثم خليط الفركتوز و الكزايللوز(7.1 مغ وزن جاف/نبات) ثم السكروز (5.4 مغ وزن جاف/نبات)، في حين توقف التجذير بوجود الفركتوز.

تفتح هذه النتائج أفاقاً جديدة على المستويين الأكاديمي فيما يخص علاقة تجديد النباتات مع تغذيتها، إضافةً إلى الناحية العملية فيما يخص اختيار السكر الواجب إضافته لوسط النمو الخالي من منظمات النمو بهدف الحصول على نباتات مطابقة للنبات الأم.

الكلمات المفتاحية: *Obione portulacoides*، الإكثار الدقيق، الكربوهيدرات، الزراعة ضمن الأنابيب الزجاجية.

Abstract

Using *In vitro* cultivated explants, of *Obione portulacoïdes* (L.) Moq was regenerated. The *Obione portulacoïdes* (L.) Moq is a halophyte shrub widely distributed in temperate Eurasia and parts of Africa, found in salty marshes and coastal dunes. This research was conducted to study, in particularly, the influence of three carbohydrate sources, i.e. sucrose, fructose and xylose (essentially in the absence of growth regulators) on different morphogenic reactions. In fact, there is a potential possibility for regenerating complete plants without any exogenous hormone.

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The highest shoots number (4.2) and highest roots number (6.3) were observed when culturing on medium supplemented with a mixture of fructose-xylose, followed by (2.9) and (4.2) respectively in presence of xylose. The observed dry weight was (9.3 mg) in presence of xylose, followed by (7.1 mg) in presence of the mixture (fructose-xylose), and (5.4 mg) when sucrose is added. The rooting was stopped in the presence of fructose. These results open numerous perspectives concerning both a fundamental approach of morphogenesis in connection with tissue nutrition and a special applied interest for the media containing a chosen hydrocarbon source, and deprived of growth regulators- free, for conform multiplication.

Keywords: *Obione portulacoides*, Micropropagation, Carbohydrates, *in vitro* culture.

Introduction

Obione portulacoïdes (L.) Moq. or sea purslane (2n=36) is a small greyish-green shrub belongs to the genus Halimione of the family Chenopodiaceae, widely distributed in temperate Eurasia and parts of Africa. A halophyte is found in saltmarshes and coastal dunes, and is usually flooded at high tide. The plant grows to 75 cm and it is evergreen. In northern temperate climates, it flowers from July to September. The flowers are monoecious and pollinated by wind. Plant is suitable for sandy, loamy and clay soils and can grow in nutritionally poor soil. Suitable pH for the plant growth is the acid, neutral and alkaline soils. Moreover, plant grows in very alkaline and saline soils. It can grow in semi-shade (light woodland) or without shade. It prefers moist or wet soils. The plant can tolerate maritime exposure (Flores and Davis, 2001).

In France, *O. portulacoïdes* grows in the salty muds near the seaside and in Camargue (south of France). It's grazed by sheep, goats or bulls in this vicinity. In winter time, *O. Portulacoïdes* represent a very important nutrition source for wild horses feeding, (Duncan, 1992). The edible leaves can be eaten raw in salads or cooked as a potherb. They are thick and succulent with a good crunchy texture and a natural saltiness (Halimione portulacoides at Plants for a Future, accessed 2012-12-14)

Since the works of El Maataoui et *al.,* (1998) and Alzubi (2002) done on the *in vitro* growth of the *Albizzia julibrissin* without any hormones, the morphogenetic development in plants in vitro without hormones has touched all kind of plant from the *Arabidopsis* (Frank et *al.,* 2000), Lemon basil, Oregano, Peppermint, Thyme (Tisserat and Silman, 2000), Orange, Lemon (Obukosia and Waithaka, 2000), Cotton (Gialvalis and Seagull, 2001), Vanilla (Giridhar et *al.,* 2001), Sidr (Sudhersan and Hussain, 2003), Maize (Shohael et *al.,* 2003), *Rotula aquatica* (Chithra et *al.,* 2004), *Dendrobium nobile* (Faria et *al.,* 2004), Rose (Kamo et *al.*, 2005), Date palm (Eke et *al.,* 2005). Some works showed that the sugar chemic nature plays a considerable role on the morphogenesis reaction at least on the semi-herbaceous models. The sugars might play a role as important as the growth regulators (Belaizi and Boxus, 1995; Romano et *al.,* 1995; Mandal and Gupta, 2001). However, the majority of studies on the influence of sugars have been realized with hormones. This fact complicates the definitive conclusion concerning the specific role of the sugars.

In this study, the in vitro growth of *O. portulacoïdes* was achieved by using three kinds of sugars (sucrose, fructose, and xylose) without addition of exogenous hormones.

Material and Methods

-Plant material

O. portulacoïdes plants, brought back from (Domaine de la Palissade, Salin-de-Giraud, Camargue - south of France) were used in this study. Ten shoot tips (0.5-0.75 cm in length) were collected per plant, including the apical meristem and adjacent axillary buds. Large leaves were removed and shoot tips were surface sterilized in a laminar flow hood by briefly washing with 70% ethanol for 30 sec to remove surface wax. Then, explants were surface sterilized for 15 min with a hypochlorite solution (10 ml commercial chlorox containing 6% w/v sodium hypochlorite diluted in 40

ml deionized water) plus 3-4 drops of Tween-20 as a wetting agent. Following sterilization, shoot tips were rinsed with sterile distilled water three times for 5 min and the base of explants (1-2 mm) were removed.

-Media and growth conditions

- **Establishment of shoot tips in tissue culture**

Sterilized shoot tips (0.5-0.75 cm in length) were established on basal (MS) Murashige and Skoog (1962) containing only macro and microelements with vitamins half strength supplemented or not with sugars at different concentrations. Individual shoots (0.5-0.75 cm in length) were sub-cultured every four weeks.

- **Multiplication and rooting media**

The initial works were allowed adopting Murashige and Skoog medium (MS) half strength to experiment the effect of chosen sugars on low concentrations. At the end of the fifth subculture, shoots of (0.5-1) cm were harvested from 4-weeks old proliferating cultures and cultured on the following media for both of multiplication and rooting of O. portulacoïdes plants.

1. MS full strength MS
2. MS half strength ½ MS
3. ½ MS + 5 sucrose $g.l^{-1}$ + 0.2 mg. l^{-1} IBA + 2 mg. l^{-1} BAP
4. ½ MS + 5 $g.l^{-1}$ sucrose
5. ½ MS + 10 $g.l^{-1}$ xylose
6. ½ MS + 5 $g.l^{-1}$ fructose
7. ½ MS + 5 $g.l^{-1}$ xylose + 2.5 $g.l^{-1}$ fructose

The first tow media were used as controls. The concentrations of IBA and BAP in the third medium correspond to those recommended by Glenn and O'Leary (1992) in order to multiply a *Chenopodiaceous neighbour*, *Salicornia bigelovii.*

The last four media were successfully used for *Albizzia Julibrissin* regeneration (Alzubi, 2002 and 2009).

After adjusting the pH to 5.6, (2 g l^{-1}) of activated charcoal were added (Collins and Dixon, 1992). Media were solidified with (6.5 g l^{-1}) agar and dispensed into glass test tubes, shut by Cap-O-Test, of 200 mm x 24 mm with 30 ml per tubes prior to autoclaving at 120°C and 1.2 kg cm^{-2} for 15 min. One shoot tip was established per test tube. Cultures were maintained at 23 ± 2°C and 80% relative humidity. They were placed under a light intensity of 60 $\mu m.m^{-2}.s^{-1}$ (Sylvania, Gro-Lux fluorescent tubes) with a photoperiod of 16 hr.

-Transfer of micropropagated plants to soil

Micropropagated plants were gently removed from test tubes and roots were rinsed in water to discard any remaining tissue culture agar. Prior to establishment on soil, roots were trimmed to about one third in length to stimulate growth. Plants were established in a mixture of peat, vermiculite, ground limestone, in plastic pots (10 cm in diameter) in a greenhouse and covered with plastic bags to avoid dehydration. Plastic bags were gradually opened following active growth (2-3 weeks) at 20 ± 5°C and 75 to 150 $\mu E\ m^{-2}.\ s^{-1}$.

-Data collection and statistical analyses

Thirty explants were used per treatment and each experiment was replicated at least twice. Shoot number, shoot length, root number, root length, root dry weight, were recorded after 30 days of culture to evaluate optimal conditions for plant development and rooting. Each culture tube or jar was a repetition in a randomized block experimental design, in which the different media were compared. Data were subjected to ANOVA to evaluate the combined effect of the different treatments and interactions using SAS® (Statistical Analysis System, version 9.1, SAS Institute Inc., Cary, NC. USA).

Results and Discussion

-Disinfection and culture initiation

Contamination *in vitro* is one of serious problems encountered tissue culture initiation. Plants growing in open conditions are frequently contaminated both externally and internally by various microorganisms. Most of these organisms are of no particular threat to the plant *in vivo*, but represent an essential obstacle to the in vitro culture, because of bacterial and fungal spores rapidly growing on the rich moderate culture.

Results showed that 1.5% w/v sodium hypochlorite solution (10 ml commercial chlorox containing 6% w/v sodium hypochlorite diluted in 40 ml deionized water plus 3-4 drops of Tween-20 as a wetting agent have a good efficiency on surface-disinfection of explants with 87% efficiency with survival rate of 90%.

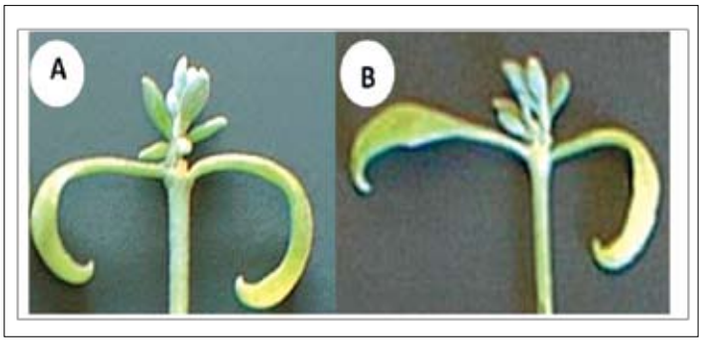

Fig1. Initial culture of *obione portulacoides* (L.) moq.
A: culture on ½ MS.
B: culture on ½ MS+5g.l-1 sucrose+0.2mg.l-IBA+2mg.l-1BAP

Lower concentrations were insufficient for explants disinfection even when used for longer time. Higher concentrations were strong enough to cause bleaching the color and the death of explants.

-Effects of used sugar on shoot organogenesis and Shoot multiplication *in vitro* of *obione portulacoides*

Browning was observed after autoclaving when fructose was added to the culture media. This might be attributed to the dehydration of products of fructose, as for example the 5-(hydroxymethyl)-2-furaldehyde, or the production of products of carbolic acids that affect the plants regeneration (Suortti, 1983; Alzubi, 2002 and 2009).

No active shoot proliferation or stem elongations were observed when explants cultured on MS medium. However, an elongation of about 3 mm in average after 30 days of culture was registered by using ½ MS medium (Fig. 1). The ½ MS was adopted to study the effect of the tested sugars. Explants elongation on a ½ MS medium supplemented with 5 $g.l^{-1}$ sucrose, 0.2 $mm.l^{-1}$ IBA and 2 $mg.l^{-1}$ BAP was 7 mm in average after 30 days of growth.

Table 1. Effect of sugar nature, added to medium on number and length of shoots produced within 4 weeks of culture.

N°	Treatment	Average of shoot number	Average of shoot length (cm)
1	MS full strength MS	0	0
2	MS half strength ½ MS	0.7 ± 0.3 ^{c}	0.3 ± 0.1^{d}
3	½ MS + 5 sucrose $g.l^{-1}$ + 0.2 mg. l^{-1} IBA + 2 mg. l^{-1} BAP	0.9 ± 0.1 ^{c}	0.4 ± 0.2^{d}
4	½ MS + 5 $g.l^{-1}$ sucrose	2.4 ± 0.4 ^{b}	2.8 ± 1.3^{c}
5	½ MS + 10 $g.l^{-1}$ xylose	2.9 ± 0.3 ^{b}	3.7 ± 1.6^{b}
6	½ MS + 5 $g.l^{-1}$ fructose	1.1±0.4^{c}	2.4 ± 0.8^{c}
7	½ MS + 5 $g.l^{-1}$ xylose + 2.5 $g.l^{-1}$ fructose	4.2 ± 0.2^{a}	4.3 ± 1.9^{a}

Notes: Data (mean±SE) from two independent experiments of 30 replications were collected after 30 days of culture.
Means within a column followed by the same letter are not significantly different.

Considering both number of shoots/explant and the length of shoots, results presented in table 1 demonstrating the effects of sugar present in culture media. The best shoot multiplication was achieved on ½ MS medium supplemented with 5 $g.l^{-1}$ xylose and 2.5 $g.l^{-1}$ fructose, with multiplication rate of 4.2 shoots/explant. Significant reductions in shoot multiplication rate were observed when the sugar mixture was replaced with whether xylose alone which resulted in a multiplication rate of 2.9 or sucrose alone with a multiplication rate of 2.4.

For shoot elongation, different elongation responses were registered depending on the added sugar.

The highest stem elongation was observed in presence of 10 $g.l^{-1}$ xylose whether used alone (3.7) cm ±1.6 cm or accompanied with 2.5 $g.l^{-1}$ fructose (4.3) cm. While the lowest stem elongation (2.8) cm ± 1.3 cm was observed in presence of either 5 $g.l^{-1}$sucrose or (2.4) cm ± 0.8 cm in presence of 5 $g.l^{-1}$ fructose ± 1.9 cm (Table 1).

However, more explant ramifications were observed in presence of the mixture fructose-xylose than that observed in presence of xylose or fructose alone. It was equivalent to the one observed in the presence of sucrose.

-Rooting

Proliferated shoot tips (0.5-1 cm length) were excised and rooted readily on ½ MS medium supplemented with the different sugars described above. Rooting was observed from the cut ends of the shoots within 30 days. Results in table 2 present rooting data, where it was shown that no rhizogenesis has been observed when the explants were cultured on MS. On ½ MS medium, 25% of rooting rate with the average number of root 1.3 per rooted explants and 1.1 cm of root length were observed. All sugars added to the culture media, except fructose, induced rooting. Developing roots were physically vigorous and healthy. The best rooting rate (96.3%) with highest average roots number of 6.3 and the average of root length of 3.6 cm were achieved on ½ MS medium + 5 $g.l^{-1}$ xylose + 2.5 $g.l^{-1}$ fructose. While, 76.6% rooting efficiency was recorded in presence of 10 $g.l^{-1}$ xylose, with average roots number of 4.2 and average of root length of 4.3 cm. The presence of 5 $g.l^{-1}$ sucrose in rooting media reduced the rooting rate to 63.6 with average roots number of 4.8 and average of root length of 2.1 cm. The explants cultivated on ½ MS medium supplemented with 5 $g.l^{-1}$ sucrose + 0.2 $mg.l^{-1}$ IBA + 2 $mg.l^{-1}$ BAP have shown necrosis at their base, followed by the death of the explants (Table 2).

Table 2. Effect of sugar nature, added to medium on number and length of shoots produced within 4 weeks of culture.

N°	Treatment	Rooting rate %	Average of root number	Average of root length (cm)	Average of D. W®. of root system
1	MS full strength MS	0	0	0	0
2	MS half strength ½ MS	25	1.3 ± 0.6 ^{C}	1.1 ± 0,3 ^{bc}	3.7 + 1.3^{c}
3	½ MS + 5 sucrose $g.l^{-1}$ + 0.2 mg. l^{-1} IBA + 2 mg. l^{-1} BAP	0	0	0	0
4	½ MS + 5 $g.l^{-1}$ sucrose	63.3	4.8 ± 2.1 ^{B}	2,1 ± 1,3 ^{b}	5.4 + 1.1b^{c}
5	½ MS + 10 $g.l^{-1}$ xylose	76.6	4.2 ± 0.7 ^{B}	4,3 ± 0,7 ^{a}	9.3 + 0.9^{a}
6	½ MS + 5 $g.l^{-1}$ fructose	0	0	0	0
7	½ MS + 5 $g.l^{-1}$ xylose + 2.5 $g.l^{-1}$ fructose	96.3	6.3 ± 0.6 ^{A}	3,6 ± 0,6 ^{a}	7.1 + 1.2^{b}

Notes: Data (mean±SE) from two independent experiments of 20 replications were collected after 30 days of culture.
Means within a column followed by the same letter are not significantly different. D.W ®. (Dry weight).

The best results, confirmed by evolution of dry weight of root system, are presented in (Table 2). The highest dry weight (9.3) mg was recorded in presence of 10 g.l^{-1} xylose, followed by (7.1) mg in presence 5 g.l^{-1} xylose + 2.5 g.l^{-1} fructose then (5.4) mg in presence of 5 g.l^{-1} sucrose in rooting media. Finally (3.7) mg on ½ MS alone (Table 2).

-Acclimatization of rooted plantlets

During the *in vitro* culture, plantlets grow under very special conditions, after transfer to the ex vitro conditions, plantlets need gradual changes in environmental conditions to avoid desiccation losses and photo inhibition.

A successful establishment in soil was achieved with a high survival rate (90%) for plants growing in presence of xylose only or with fructose. In contrast, establishment in the greenhouse was poor (35%) for plants growing in presence of sucrose due to poor rooting (Fig.2).

The results show that the sugar present in the growth medium plays a remarkable key role in morphogenesis evolution while orientating this one. The regulator effect of the used sugars confirms the results obtained by Alonso-Lopez (1996); Alzubi (2002 and 2009) using similar sugars for *Albizzia Julibrissin* regeneration from root or hypocotyls segments.

The explants show a remarkably precocious potentiality of caliginosity in absence of hormonal means, whatever the sugar is used. Our results are the confirmation of the stimulative effect of the fructose-xylose or of the xylose on the formation of the buds, without any hormones in the medium (Romano et *al.,* 1995; Alsonso-Lopez, 1996; Alzubi, 2002 and 2009).

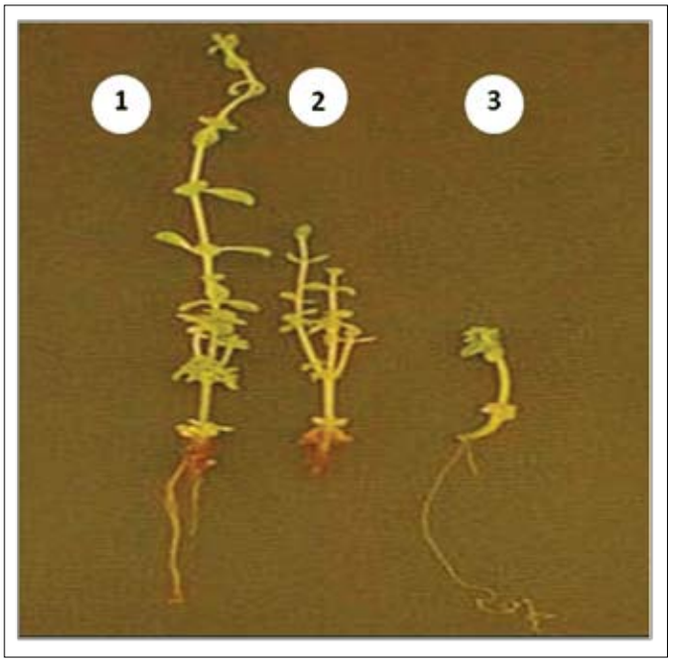

Fig.2. Effects of sugar nature on *in vitro Obione portulacoïdes* regeneration. Plants are induced and rooted on:

1: ½ MS + 5 g.l^{-1} xylose + 2.5 g.l^{-1} fructose,

2: ½ MS + 10 g.l^{-1} xylose,

3: ½ MS + 5 g.l^{-1} sucrose.

Results show that the rooting *in vitro* does not need an elevated concentration in mineral salts (Bon et *al.,* 1998; Kromer and Gamian, 2000). They indicate that the regulators of growth and vitamins are of no use for the rooting. (Kooï et *al.,* 1999) have shown that the augmentation of the concentration in sucrose of the medium from 15 g/l to 30 g/l delayed the formation and the frequency of apparition of roots on the leafy shoots of *Azadirachta excelsa* In our study, the concentrations which were enabling the rooting were indeed low, such as 5 g.l^{-1} sucrose, 10 g.l^{-1} xylose and 2.5 g.l^{-1} fructose + 5 g.l^{-1} xylose. It might correspond to the regulator role of the sugars on the expression of auxins (Ryan and Farmer, 1991). The xylose was the best for the rooting which agree with results obtained by Jain and Babbar (2003) using epicotyl explants of *Zyzygium cuminii* to study the effect of carbon source on the shoot proliferation. It is found that in presence of experimented sugars in low concentrations and in absence of any exogenous hormones, the leafy shoots induced on the corresponding sugars have better rooting. These low concentrations in sugars should be linked to an inside hormonal regulation.

These intracellular carbohydrates must indeed play a biologic role different from this simple energetic source (Ryan and Farmer, 1991). They might intervene in the regulation of the organogenesis (Trinh et *al.,* 1990), or in the regulation of the rooting (Vasseur et *al.,* 1987; Alzubi, 2002 and 2009).

Conclusions

The originality in this study is the possibility of regenerating complete plants using three carbohydrate sources, of. sucrose, fructose and xylose, without any exogenous growth regulators. The use of xylose, generally unused in tissue culture, has been demonstrated, essentially in rooting, which gave the best results. It appears primordial that the mechanisms of the action of sugars on the morphogenesis must be precise and put into relation with those attributed to the presence of hormones, and particularly of auxins.

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