Effects of the methanolic extracts of six cactus pear species (*Opuntia* spp.) on tissue browning and endophytic bacteria of date palm (*Phoenix dactylifera* L.)

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Abstract

Organogenesis is the in vitro regeneration technique used in Morocco for rapid and large-scale propagation of date palm. Unfortunately, this technique is hampered by tissue browning, a physiological disorder that decreases the proliferation capacity of shoot buds and affects the quality of the multiplied plants, and by endophytic bacteria, which are introduced in vitro even if explants are well disinfected. In order to solve these problems, the effects of different concentrations of the methanolic extracts from six *Opuntia* species were evaluated during shoot bud multiplication of date palm cv. Mejhoul. Organogenic cultures of date palm cv. Mejhoul were cultured for 4 months on semi-solid and half-strength Murashige and Skoog (MS/2) medium, supplemented with plant growth regulators (PGRs) and different concentrations (1, 5 or 10 ml/l) of the methanolic extracts from cladodes of *Opuntia megacantha*, *O. ficus indica*, *O. robusta, O. aequatorialis*, *O. leucotricha* and *O. inermis*. Our results showed that, regardless of the cactus pear species used, the concentration of 10 ml/l of the methanolic extract is not suitable for shoot bud multiplication since it results in considerable phytotoxicity. The optimal concentration for shoot bud multiplication was 1 ml/l of the extracts of *O. ficus indica*, which showed the highest number of shoot buds per explant (14.2), 5% tissue browning and the elimination of endophytic bacteria. The use of the methanolic extracts from cactus pear petals also resulted in bacterium elimination. However, it did not improve the multiplication rate. After the multiplication phase, shoot buds were transferred to the elongation-rooting medium then to glasshouse where a high survival rate of 80% was observed.

Keywords: Phoenix dactylifera L.; Organogenesis; Methanolic extracts; Opuntia spp.; Tissue browning; Endophytic bacteria.

Effets des extraits méthanoliques de six espèces du cactus (*Opuntia* spp.) sur le brunissement des tissus et les bactéries endophytes du palmier dattier (*Phoenix dactylifera* L.)

Résumé

L'organogenèse est la technique de micropropagation utilisée au Maroc pour la multiplication rapide et massive du palmier dattier. Malheureusement, cette technique est entravée par le brunissement des tissus, un désordre physiologique qui limite la prolifération des bourgeons adventifs et affecte la qualité des plantules multipliées ; et la contamination par les bactéries endophytes qui apparaissent même si les explants sont bien désinfectés. Afin de résoudre ces problèmes, nous avons évalué l'effet des extraits méthanoliques de six espèces d'*Opuntia* sur la multiplication des bourgeons adventifs du palmier cv. Mejhoul. Ainsi, des souches bourgeonnantes du palmier dattier cv. Mejhoul ont été cultivées pendant 4 mois sur le milieu semi-solide de Murashige et Skoog, dilué de moitié (MS/2), additionnés de régulateurs de croissance et de différentes concentrations (1 ; 5 ou 10 ml/l) d'extraits méthanoliques de cladodes d'*Opuntia megacantha, O. ficus indica*, *O. robusta*, *O. aequatorialis*, *O. leucotricha* and *O. inermis*. Nos résultats ont montré que, quel que soit le matériel végétal utilisé, la concentration de 10 ml/l de l'extrait méthanolique entraîne une phytotoxicité accentuée. Par ailleurs, le traitement optimal pour la multiplication des bourgeons est 1 ml/l d'extraits de cladode d'*O. ficus indica*. Ce traitement a montré le taux de multiplication le plus élevé (14.2 bourgeons par explant), le taux de brunissement le plus faible (5%) et la disparition totale de la bactérie. D'un autre côté, l'utilisation des extraits méthanoliques des pétales a également éliminé la bactérie. Toutefois, ces extraits n'ont pas amélioré le taux de multiplication des bourgeons. Les bourgeons ont été ensuite transférés sur le milieu d'élongation-enracinement puis sous serre où un taux de survie de 80% a été observé.

Mots clés : Phoenix dactylifera L.; Organogenèse; Extraits méthanoliques; Opuntia spp.; Brunissement des tissus; Bactéries endophytes.

تأثير مستخلصات الميثانول لستة أنواع من شجر الصبار (*Opuntia* spp.) على اسمرار الأنسجة والبكتيريا الداخلية لنخيل الثمر(*Phoenix dactylifera* L.)

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ملخص

تعتبر تقنية التكوين العضوي داخل الأنابيب الطريقة الأكثر استعمالا في المغرب من أجل التكاثر السريع لنخيل الثمر، إلا أن عملية تكاثر البراعم بواسطة هذه التقنية تعرف عدة مشاكل من بينها اسمرار الأنسجة، وهو اضطراب فسيولوجي يقلل من معدل التكاثر ويؤثر على جودة الشتلات المنتجة؛ والتلوث بالبكتيريا الداخلية، التي تظهر في وسط النمو وبمحاذاة الأنسجة حتى بعد عملية التعقيم. من أجل حل هذه المشاكل، قمنا بدراسة تأثير مستخلصات الميثانول من ستة أصناف من شجر الصبار على تكاثر براعم نخيل الثمر صنف المجهول. في هذا الإطار، تمت زراعة الأنسجة البرعمية لصنف المجهول على الوسط شبه الصلب المخفف Murashige و Skoog (MS/2) و المحتوي على هرمونات النمو، إضافة إلى تراكيز مختلفة (1 ؛ 5 أو 10 مل/لتر) من مستخلصات الميثانول لستة أنواع من الصبار : *Opuntia megacantha* ، *O. ficus indica* ، *O. robusta* ،*O. aequatorialis* ،*O. leucotricha* و *O. inermis*.أظهرت نتائج هذه التجارب أنه بغض النظر عن مصدر المستخلصات المستخدمة، فإن تركيز 10 مل/لتر كان ساما لأنسجة نخيل الثمر. أظهرت نتائجنا أيضا أن التركيز الأمثل لتكاثر البراعم هو 1 مل/لتر من مستخلصات نوع *O. ficus indica*، والذي أنتج أعلى معدل تكاثر (14.2 برعم)، أدنى معدل اسمرار (5٪) إضافة إلى قضائه كليا على البكتريا الداخلية لأنسجة نخيل الثمر. من ناحية أخرى، أظهر استخدام مستخلصات الميثانول من بتلات *O. ficus indica* اختفاء تام للبكتريا الداخلية لأنسجة نخيل الثمر، إلا أن هذه المستخلصات لم تحسن من معدل تكاثر البراعم. بعد مرحلة التكاثر، تم نقل البراعم إلى وسط الاستطالة والتجذير ثم إلى البيت الزجاجي حيث 80% أظهرت نموا طبيعيا.

الكلمات المفتاح: *Phoenix dactylifera L. ؛ تكوين الأعضاء ؛ مستخلصات الميثانول ؛ Opuntia؛ اسمرار الأنسجة ؛ بكتيريا داخلية.*

Introduction

Organogenesis is the in vitro regeneration process in which adventitious buds and roots are induced to develop and form complete plants. This regeneration system comprises the following steps: adventitious bud induction, shoot bud multiplication, shoot elongation and rooting and finally plantlet acclimatization (Mazri and Meziani, 2013). In date palm (*Phoenix dactylifera* L.), organogenesis has been used for large-scale propagation of two categories of cultivars: (i) the best date palm cultivars that are highly demanded by farmers and consumers, but unfortunately are threatened by the bayoud disease, which is a wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *albedinis* and that killed millions of date palm plants during the last century in Morocco and Algeria (Jaiti et al., 2007). In this case, the regenerants are planted in bayoud-free areas (Mazri, 2015; Mazri et al., 2016). Examples of these cultivars are Mejhoul, Boufeggous and Bouskri. And (ii), the cultivars resistant to the bayoud disease and characterized by high fruit quality (Mazri and Meziani, 2013; Mazri, 2019). These cultivars were selected by the researchers of the National Institute of Agronomic Research of Morocco (INRA) and are now used to rehabilitate date palm groves infested by bayoud. Examples of these cultivars are Najda, Al-Fayda and Sedrat (Sedra, 2011).

Developing an efficient regeneration system through organogenesis for date palm is hampered by many factors, two of the most important among them and that may cause drastic losses during the regeneration process are tissue browning and contamination with the endophytic bacteria associated with date palm explants. Browning of date palm explants occurs due to the important level of caffeoylshikimic acids in date palm tissues (Loutfi and El Hadrami, 2005) while the endophytic bacteria are difficult to eliminate through surface sterilization and could appear even after several subcultures (Meziani et al., 2019a). Thus, in the recent years, efforts have been made to find solutions to these problems (Mazri, 2015; Mazri et al., 2016; Meziani et al., 2016; 2019a). For example, the effect of different synthetic and natural compounds on tissue browning was evaluated, namely date stone-based activated carbon (DSAC), polyvinylpyrrolidone (PVP), activated charcoal, as well as the methanolic extracts from *Rosmarinus officinalis* and *Thymus satureioides* (Meziani et al., 2016). On the other hand, the most frequently endophytic bacteria encountered during in vitro culture of date palm were identified by using 16S rRNA gene amplification and sequencing as *Microbacterium testaceum* and *Serratia marcescens*, and different methods to control them were tested (Meziani et al., 2019a). Based on these experiments, it was demonstrated that some natural compounds, such as DSAC and the essential oils of *Artemisia herba-alba*, could replace PVP and antibiotics, respectively, which are widely used and cost a lot. Accordingly, more investigations should be carried out in order to determine natural products characterized by both antioxidant and antibacterial properties, and thus able to reduce tissue browning and to control the endophytic bacteria associated with date palm explants.

Cactus pear (*Opuntia* spp.) is a plant genus cultivated in many regions of the world for different purposes. For example, cactus pear is used for human consumption, to feed livestock and for nutraceutical applications (Mazri, 2018). In fact, many researchers highlighted the antioxidant and antibacterial activities of cactus pear. For instance, Kuti (2004) indicated the abundance of antioxidant compounds in the fruits of four cactus pear species (*Opuntia ficus indica*, *O. lindheimeri*, *O. streptacantha* and *O. stricta*)

while Santos-Zea et al. (2011) reported the high antioxidant activity of cladodes of different cactus pear species (*O. ficus indica*, *O. lindheimeri*, *O. robusta*, *O. streptacantha*, *O. undulata*, *O. rastrera* and *O. leucotricha*). In addition, Sánchez et al. (2014) demonstrated the antibacterial activity of the methanolic extracts from cladodes of *O. ficus indica* cultivars against *Campylobacter Jejuni*, *Vibrio cholera* and *Clostridium Perfringens*.

The purpose of the present investigation was to evaluate the effect of the methanolic extracts from six cactus pear species, namely *O. megacantha, O. ficus indica*, *O. robusta*, *O. aequatorialis*, *O. leucotricha* and *O. inermis*, at different concentrations (1, 5 or 10 ml/l), on shoot bud multiplication and tissue browning of date palm. Besides, we assessed their efficiency in controlling the endophytic bacterium (*Microbacterium testaceum*) associated with date palm explants.

Materials and methods

Preparation of methanolic extracts from *Opuntia* spp.

Cladodes of two-year-old plants of six *Opuntia* species, namely *O. megacantha, O. ficus indica*, *O. robusta*, *O. aequatorialis*, *O. leucotricha* and *O. inermis* were used to prepare the methanolic extracts. The cladodes were collected from the experimental station Ain Zagh of the National Institute of Agronomic Research of Settat (INRA, Settat, Morocco). Among the studied species, *O. ficus indica* and *O. inermis* are spineless.

The cactus cladodes were dried in the oven (XU980, France-Etuves, Chelles, France) at 55°C for 12h then ground to powder. For each plant material, 10 g of fine powder (20 mesh) was macerated with 100 ml methanol (Sigma, St. Louis, MO, USA) for 5h at room temperature. The macerate was filtered then centrifuged at 3000*g* for 20 min at room temperature. The extracts were filtered then stored at 4°C in darkness until use.

Effect of *Opuntia* methanolic extracts on date palm organogenesis

Offshoots of date palm (*Phoenix dactylifera* L.) cv. Mejhoul were collected from Erfoud, Morocco. The shoot tips were removed, disinfected and organogenic cultures were induced according to the protocol of Mazri et al. (2018). All the organogenic cultures used in the present investigation showed the presence of the endophytic bacterium *Microbacterium testaceum*, which appears in the culture medium and around explants as a transparent white zone. The culture medium used in the present study was that of Murashige and Skoog at half-strength (MS/2; Murashige and Skoog, 1962), supplemented with 30 g/L sucrose, 6 g/L agar, 0.9 µM 2-naphthoxyacetic acid (NOA), 1.1 µM indole-3-acetic acid (IAA), 1.8 µM kinetin and 1.9 µM 6-(dimethylallylamino) purine (2iP) as suggested by Meziani et al. (2015). The pH of all media was set to 5.7 before autoclaving at 121 °C for 20 min.

After autoclaving, different concentrations (1, 5 or 10 ml/l) of the methanolic extracts from the cladodes of the six above-mentioned cactus pear species were added to culture medium. The methanolic extracts were added by using Millipore filters. The cultures were kept for 4 months under a 16h photoperiod and 25°C, and transferred to a fresh medium at 1-month-intervals. Shoot bud multiplication was assessed by calculating the average number of shoot buds per explant and determining the total protein content in the organogenic cultures. For each treatment, 10 replicates were used. As a control, a medium without cactus pear extracts was used.

As a second experiment, methanolic extracts (1, 5 and 10 ml/l) of petals of *O. ficus indica* were prepared and added to culture medium following the same above-mentioned protocol. The cultures were kept for 4 months under the same-above mentioned conditions. For each treatment, 10 replicates were used. The findings of this experiment were compared to those of the best treatment from the first assay.
The developed shoots were transferred to semi-solid and plant growth regulator (PGR)-free MS/2 medium containing 30 g/L sucrose for elongation and rooting (16h photoperiod, 25 °C) as suggested by Meziani et al. (2019b). After 3 months of culture, the shoots were transferred to the glasshouse according to the protocol described by Mazri et al. (2018).

Total protein determination in date palm organogenic cultures

After 4 months of culture, the total protein content was determined according to the protocol described by Meziani et al. (2016). Briefly, Organogenic cultures (3 g fresh weight (FW)) were pounded in 10 ml of Tris–HCl buffer (50 mM, pH 8) and agitated. Afterwards, the mix was incubated for 24h at 4 °C. The mix was then centrifuged at 13,000*g* for 15 min and the supernatant was separated and subjected to protein measurements according to Bradford (1976). For each treatment, 10 replicates were used.

Peroxidase activity determination in date palm organogenic cultures

After 4 months of culture, the total peroxidase activity was determined according to the protocol described by Meziani et al. (2016). Briefly, Organogenic cultures (250 mg FW) were pounded in 2 ml of cold 0.1 M Tris-maleate buffer then the mix was agitated and centrifuged at 13,000*g* for 15 min. the supernatant was separated and subjected to peroxidase activity measurements according to Jaiti et al. (2009). For each treatment, 10 replicates were used.

Data collection and statistical analysis

At the end of multiplication experiments, the following data were recorded: explant phytotoxicity (determined visually and reflects the organogenic cultures that became necrotic and died), the average number of shoot buds per explant, the percentage of tissue browning, the browning intensity (visually estimated as low, moderate and high), the presence/absence of *Microbacterium testaceum* (determined visually by checking in the culture medium and around explants), total protein content (which reflects the vigor of adventitious shoot buds) and the peroxidase activity of explants.
In all experiments, we placed 4 organogenic cultures per jar. The experiments were conducted following a completely randomized design. The data were subjected to

ANOVA ($P<0.05$) and the means were separated by using the Student-Newman-Keuls test at the 5% significance level. Calculations were made by SPSS for windows (v. 26). Before analysis, all percentage data were arcsine transformed.

Results and Discussion

Many plant extracts were reported to have biological activities and were suggested for different applications. The antibacterial and antioxidant activity of cactus pear species was scientifically demonstrated. Indeed, Kuti (2004) and Santos-Zea et al. (2011) reported that cactus pear fruits and cladodes are characterized by a high antioxidant activity whereas Sánchez et al. (2014) reported that the methanolic extracts of cactus pear cladodes have a potent antibacterial activity. However, the use of cactus pear extracts to overcome the problems that hamper date palm micropropagation such as tissue browning and contamination with the endophytic bacteria has never been assessed. In the present study, we evaluated the effects of different concentrations of methanolic extracts from six cactus pear species on tissue browning and endophytic bacteria of date palm during organogenesis. Our results showed that adding cactus pear methanolic extracts at the concentration of 10 ml/l was toxic to organogenic cultures, regardless of the species used (Fig. 1; Table 1). This is in good agreement with the findings of Meziani et al. (2019a), which showed that the concentration of 1% of some plant extracts is toxic to date palm explants. Thus, this concentration is not recommended. The phytotoxicity of the concentration of 5 ml/l depended on cactus pear species. In fact, this concentration was not toxic in the case of *O. ficus indica* and *O. megacantha*. Regarding the concentration of 1 ml/l of the methanolic extracts of cactus pear, it did not show any phytotoxic effect on the organogenic cultures of date palm (Table 1).

Figure 1: Phytotoxicity of the explants cultured on media containing 10 ml/l of the methanolic extracts of cactus pear

The use of the methanolic extracts of cactus pear at the concentration of 1 ml/l showed very interesting results against tissue browning. Indeed, tissue browning ranged from 5 to 25% (Table 1). The lowest tissue browning percentage was observed in the culture medium containing 1 ml/l methanolic extracts of *O. ficus indica*. This was followed by the concentration of 1 ml/l *O. megacantha*, which showed a tissue browning percentage of 10% (Table 1). The use of the methanolic extracts of *O. ficus indica* and *O. megacantha* at the concentration of 5 ml/l showed a tissue browning percentage of 15%. The highest tissue browning percentage (25%) was observed when the methanolic extracts of *O. inermis* were used. Statistical analysis showed no significant difference among the different treatments (Table 1). On the other hand, tissue browning was of a low intensity (i.e. affecting less than 33% of the explant). Browning of date palm explants is due to their high content in caffeoylshikimic acids (Loutfi and El Hadrami, 2005). Previous studies on date palm organogenesis reported that tissue browning is one of the main factors that may hamper commercial production of plants through this regeneration system as it can cause drastic losses during adventitious bud initiation and shoot bud multiplication (Meziani et al., 2016). Accordingly, it seems that adding an antioxidant compound to culture medium is mandatory for successful regeneration of date palm through organogenesis. In fact, the use of a medium without antioxidant additives showed a high percentage of tissue browning (95%, Meziani et al., 2016). Activated charcoal and PVP are by far the most widely used compounds during date palm organogenesis. According to Meziani et al. (2016), the use of PVP at a concentration ranging from 1.5 to 4.5 g/L showed a tissue browning percentage of cv. Mejhoul explants of 17.5-20%, while the use of activated charcoal at the same range showed a tissue browning percentage of 62.5-65%. In addition, other additives and plant extracts such as the methanolic extracts of *Rosmarinus officinalis* and *Thymus satureioides* were also evaluated against tissue browning of date palm explants. However, the use of DSAC at 1.5 g/L showed the most interesting results (7.5% tissue browning) and was thus suggested by Meziani et al. (2016). Our findings showed that the use of the methanolic extracts of *O. ficus indica* at 1 ml/l could also be suggested as it showed a very low browning percentage of 5% (Table 1).

Our findings showed that the peroxidase activity of organogenic cultures ranged from 236 to 408 enzymatic unit (U)/g FW. The lowest peroxidase activity was observed in the explants cultured on the medium containing 1 ml/l of the methanolic extracts of *O. ficus indica*, while the highest peroxidase activity was observed in the explants cultured on the medium containing 5 ml/l of the methanolic extracts of *O. megacantha* (Table 1). Our findings showed also that increasing the methanolic extracts of *O. ficus indica* and *O. megacantha* to 5 ml/L increased the peroxidase activity in explants. Peroxidase refers to a phenol-oxidizing enzyme involved in plant cell division, differentiation and growth (Genkov and Ivanova, 1995; Tang and Newton, 2005). Previous studies showed the presence of a positive correlation between the peroxidase activity of explants and tissue browning (Meziani et al., 2016). Accordingly, the concentration of 1 ml/l of the methanolic extracts of cactus pear cladodes is the most appropriate for date palm organogenesis.

The endophytic bacteria associated with date palm explants can be considered as one of the major problems that hamper the development of efficient in vitro regeneration systems through organogenesis and somatic embryogenesis. In fact, the endophytic bacteria may survive to rigorous disinfection and can appear and propagate after several subcultures, which generally leads to important losses for commercial laboratories (Meziani et al., 2019a). In a previous study, the two main endophytic bacteria associated with date palm explants were identified by using 16S rRNA gene amplification and sequencing (Meziani et al., 2019a). These bacteria are *Microbacterium testaceum* and *Serratia marcescens*, which appears as transparent white and white-pink zones, respectively. In addition, different techniques were evaluated to control them, namely by supplementing the culture medium with plant extracts, by immersing the explants in plant extracts and by soaking sterile cotton plugs with plant extracts. However, only the first method showed interesting results and thus was suggested (Meziani et al., 2019a). Accordingly, in this investigation, the methanolic extracts were incorporated into culture media. Besides, only *Microbacterium testaceum* was used in the present investigation since none of our cultures were contaminated with *Serratia marcescens*.

Table 1: Effect of the methanolic extracts from the cladodes of different cactus pear species on tissue browning and contamination with the endophytic bacteria of date palm explants

Cactus pear species	Methanolic extract concentration (ml/l)	Phytotoxicity (%)	Average number of shoot buds per explant	Tissue browning (%)	Browning intensity	Explant contamination with *Microbacterium testaceum* (%)	Total protein content (mg/g FW)	Peroxidase activity (U/g FW)
-	-	-	-	-	-	100 a	-	-
O. megacantha	1	0	13.3 ± 1.8 b	10.0 ± 21.0 a	+	0 b	76.0 ± 11.7 b	263.0 ± 87.3 a
	5	0	10.1 ± 1.7 a	15.0 ± 24.1 a	+	0 b	47.9 ± 12.8 a	408.0 ± 70.8 c
	10	100	-	-	-	0 b	-	-
O. ficus indica	1	0	14.2 ± 1.9 b	5.0 ± 10.5 a	+	0 b	81.4 ± 7.4 b	236.0 ± 61.6 a
	5	0	10.4 ± 0.9 a	15.0 ± 17.4 a	+	0 b	53.5 ± 7.4 a	403.0 ± 76.4 c
	10	100	-	-	-	0 b	-	-
O. robusta	1	0	12.9 ± 2.3 b	20.0 ± 19.7 a	+	0 b	74.8 ± 14.4 b	321.0 ± 53.2 b
	5	100	-	-	-	0 b	-	-
	10	100	-	-	-	0 b	-	-
O. aequatorialis	1	0	13.4 ± 1.5 b	22.5 ± 18.4 a	+	0 b	70.4 ± 15.2 b	350.0 ± 55.5 bc
	5	100	-	-	-	0 b	-	-
	10	100	-	-	-	0 b	-	-
O. leucotricha	1	0	12.5 ± 1.5 b	20.0 ± 22.9 a	+	0 b	71.9 ± 13.1 b	343 ± 23.5 bc
	5	100	-	-	-	0 b	-	-
	10	100	-	-	-	0 b	-	-
O. inermis	1	0	12.8 ± 0.9 b	25.0 ± 23.5 a	+	0 b	68.8 ± 17.1 b	369.0 ± 60.2 bc
	5	100	-	-	-	0 b	-	-
	10	100	-	-	-	0 b	-	-

Data are means ± standard deviation. Data in the same column followed by the same letter are not significantly different at the 5% significant level of Student-Newman-Keuls. Tissue browning intensity was visually estimated at low (+), moderate (++) and high (+++).

Our findings showed that all the cactus extracts used resulted in a complete disappearance of this endophytic bacterium, while this bacterium remained in the culture medium devoid of cactus pear extracts (Table 1). However, the high concentrations of the extracts caused severe explant phytotoxicity (Fig. 1). Thus, we recommend to use the methanolic extracts of cactus pear at low concentrations.

Our findings showed that the methanolic extracts of cactus pear affected shoot bud multiplication, and that this effect varies among the different species evaluated and depending on the concentration used. The highest average number of shoot buds per explant (14.2) was observed on the medium supplemented with 1 ml/l of the methanolic extracts of *O. ficus indica*, with no significant difference with all the other culture media supplemented with the same concentration of the methanolic extracts, regardless of the cactus pear species used (12.5-13.4 shoot buds per explant; Table 1). On the other hand, the use of the methanolic extracts of *O. ficus indica* and *O. megacantha* at the concentration of 5 ml/l showed significantly lower number of shoot buds per explant (10.1-10.4). Thus, the concentration of 1 ml/l of the methanolic extracts of cactus pear is the most appropriate for date palm cv. Mejhoul organogenesis (Fig. 2). Previous studies showed that shoot bud multiplication of date palm cv. Mejhoul depends strongly on the medium components and culture conditions, i.e. PGRs, mineral salts, carbon source, L-glutamine, myo-inositol, antioxidants and light intensity (Mazri et al., 2016; Meziani et al., 2015, 2016).

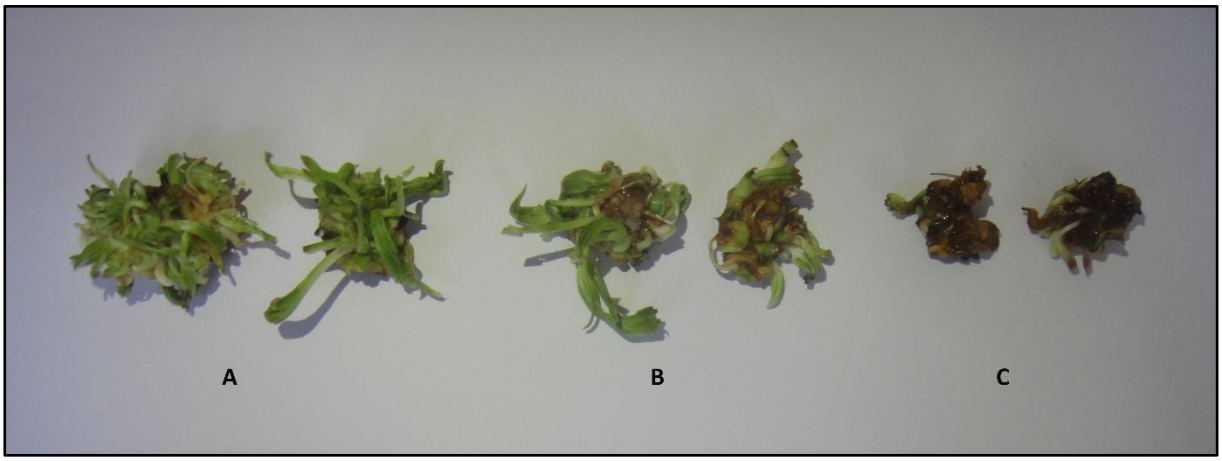

Figure 2: Effect of different concentrations of the methanolic extracts of *O. ficus indica* on shoot bud multiplication and tissue browning of date palm cv. Mejhoul. **A** Shoot buds cultured on the medium containing 1 ml/l of the methanolic extracts. **B** Shoot buds cultured on the medium containing 5 ml/l of the methanolic extracts. **C** Shoot buds cultured on the medium containing 10 ml/l of the methanolic extracts.

The results of the present investigation showed that the total protein content in organogenic cultures ranged from 47.9 to 81.4 mg/g FW. The highest total protein content was observed in the explants cultured on the medium containing 1 ml/l methanolic extracts of *O. ficus indica* (Table 1). This indicates that the concentration of 1 ml/l of the methanolic extracts from *O. ficus indica* is the most appropriate for shoot bud multiplication of date palm cv. Mejhoul. In fact, previous studies showed that high protein content in the organogenic cultures of date palm is reflected in thick and vigorous shoot buds (Meziani et al., 2016). According to Zouine et al. (2005), the

protein content in date palm tissues cultured in vitro varies depending on the culture medium composition. Besides, it was reported that high total protein content is an indicator of the embryogenic potential of date palm callus (El Hadramiand Baaziz, 1995), and characterizes somatic embryos with good cellular proliferation (Abohatem et al., 2011).

Our findings revealed high similarity between the effects of *O. ficus indica* and *O. megacantha*. According to EL Kharrassi et al. (2017), there is a high genetic similarity between these two species of cactus pear. Furthermore, Labra et al. (2003) reported that *O. ficus indica* is a domesticated form of *O. megacantha*. This may explain the high similarity observed in the present investigation between the effects of the methanolic extracts of these two cactus pear species.

In a second experiment, we compared the best treatment from the first assay (i.e. 1ml/l of the methanolic extracts from cladodes of *O. ficus indica*) with the methanolic extracts of petals of the same cactus pear species. It was found that the use of petal methanolic extracts at 10 ml/l resulted in phytotoxicity, and that the concentrations of 1 and 5 ml/l did not improve shoot bud multiplication, but instead resulted in higher tissue browning percentages (Table 2). Thus, the methanolic extracts obtained from the petals of *O. ficus indica* are not recommended for date palm organogenesis. Besides, our results showed significant differences between the effects of extracts obtained from cladodes and those obtained from petals on the peroxidase activity and total protein content in organogenic cultures (Table 2).

At the end of the multiplication experiments, the organogenic cultures were transferred to the elongation-rooting medium (PGR-free MS/2) then to the glasshouse, where a high survival rate of 80% was observed.

Table 2 Effect of the methanolic extracts from petals of *O. ficus indica* on tissue browning and contamination with the endophytic bacteria of date palm explants

Plant material	Methanolic extract concentration (ml/l)	Phytotoxicity (%)	Average number of shoot buds per explant	Tissue browning (%)	Browning intensity	Explant contamination with *Microbacterium testaceum* (%)	Total protein content (mg/g FW)	Peroxidase activity (U/g FW)
Cladodes of *O. ficus indica*	1	0	14.2 ± 1.9 b	5.0 ± 10.5 a	+	0	81.4 ± 7.4 b	236.0 ± 61.6 a
Petals of *O. ficus indica*	1	0	13.0 ± 1.4 b	30.0 ± 15.8 b	+	0	69.7 ± 11.8 a	441.0 ± 94.5 b
	5	0	10.8 ± 1.3 a	20.0 ± 34.9 ab	+	0	67.2 ± 11.6 a	419.0 ± 90.8 b
	10	100	-	-	-	0	-	-

Data are means ± standard deviation. Data in the same column followed by the same letter are not significantly different at the 5% significant level of Student-Newman-Keuls. Tissue browning intensity was visually estimated at low (+), moderate (++) and high (+++).

Conclusions

We evaluated the effects of the methanolic extracts from different cactus pear species on tissue browning and the endophytic bacteria associated with date palm explants. The methanolic extracts of *O. ficus indica* at the concentration of 1 ml/l showed the highest number of shoot buds per explant, the lowest rate of tissue browning and caused *Microbacterium testaceum* elimination. Besides, the shoots exhibited normal growth and development. Our findings showed that the use of the methanolic extracts of *O. ficus indica* could be envisaged as an efficient means to control tissue browning and bacterial contamination in date palm cultures. We are now evaluating the biochemical composition of the methanolic extracts used in this study.

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