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In vitro regeneration of two northern Moroccan *Opuntia ficus-indica* (L.) Mill. genotypes

H. Bougdaoua, N. El Mtili

Sciences de l'Alimentation et Santé, Laboratoire de Biologie et Santé, Faculté des Sciences, Tétouan

Abstract

This study focused on the valorization of seeds from two varieties (genotypes) of *Opuntia ficus-indica* harvested from the Al-Hoceima (Dellahia) and Tétouan regions of Morocco. This was achieved using *in vitro* cultivation (germination, micro-cuttings, and rooting). Seeds ≥ 5 mm in length were disinfected with 0.01% mercury chloride and cultured in a basal Murashige and Skoog medium (MS). The germinated seedlings were then proliferated in MS medium supplemented with different concentrations of benzyladenine (BAP) (0.1, 0.5, 1, and 2 mg/L). The generated shoots were then rooted in MS 1/2 medium supplemented with 30 g / L sucrose in the absence and presence of indolebutyric acid (IBA) (0.1, 0.2, and 0.5 mg/L). Dark rooting in the presence of IBA was also investigated. We achieved a germination rate of 57% and 55% for the Al-Hoceima and Tétouan varieties, respectively. We also found that darkness promoted rooting in the presence of IBA. Notably, the two varieties responded differently to media conditions, indicating the importance of optimizing the growing conditions to maximize the value of different cultivars.

Keywords: *Opuntia ficus-indica*, valorization, *in vitro* culture, seeds, Morocco.

Introduction

Opuntia ficus-indica (L.) Mill. is among the most agronomically important cacti (Kiesling, 1995). This species is native to the arid and semi-arid regions of Mexico and was introduced to North Africa in the 16th century (Griffith, 2004). It is a multi-purpose plant with diverse applications, including human food, fodder, medicine, and ornamentation (Russell & Felker, 1987). *Opuntia* species have a specialized photosynthetic mechanism, crassulacean acid metabolism (CAM), which is characterized by reduced water loss (Nobel *et al*., 1995).

Despite the presence of *O. ficus-indica* across the majority of Moroccan landscapes, its primary method of propagation is vegetative, limiting the exploitation of its potential genetic diversity. An alternative method of propagation that maintains genetic diversity is seed multiplication (Rojas-Aréchiga & Vázquez-Yanes, 2000), which has been successfully performed using *Pelecyphora aselliformis* Ehrenb. (Santos-Diaz *et al.*, 2003), eight *Turbinicarpus* species (Dávila-Figueroa *et al.*, 2005), and *Pilosocereus robinii* (Quiala *et al.*, 2009). This study sought to exploit the genetic diversity of *O. ficus-indica* using *in vitro* cultured seeds from two varieties harvested from two provinces of northern Morocco (Tétouan and Al-Hoceima). The *in vitro* germination rate and effects of benzyladenine (BAP) on shoot growth and indolebutyric acid (IBA) and darkness on rooting were evaluated.

Material and methods

O. ficus-indica seeds were obtained from ripe fruits harvested from the provinces of Tétouan and Al-Hoceima. The fruits were crushed with a sufficient quantity of water to ensure the grains and juice could be adequately separated using a

CONTACT N. El Mtili ✉ n.elmtili@uae.ac.ma 🖃 Department of Biology, Faculty of Sciences, Abdelmalek Essaâdi University, Tetouan 93000 Morocco

strainer. The seeds were then washed with water and dried under ambient conditions prior to cultivation.

All media were prepared using standard protocols with the pH adjusted to 5.7 and autoclaved at 121 °C for 15 min.

Disinfection and germination of seeds

Seeds ≥ 5 mm in length (n=40) were disinfected by immersion in 0.01% mercury chloride (w / v) for 20 min and rinsed three times with sterile distilled water. The end of each seed was then cut with a scalpel to promote germination. The seeds were placed in petri dishes with Murashige & Skoog (MS) basal culture medium (Murashige & Skoog, 1962) supplemented with 30 g / L sucrose and solidified with 7 g / L agar. The petri dishes were then incubated in a culture chamber at 26 °C with a photoperiod of 16 h light and 8 h darkness. Seed germination was assessed at 3 d intervals for five weeks.

Multiplication of shoots

Germinated seedlings (2 cm shoots) were used for the multiplication phase. The explants (n=25) were cultivated in proliferation medium containing MS supplemented with different concentrations of BAP (0.1, 0.5, 1, and 2 mg/L). After culturing for 4 weeks, the number and average length of shoots produced by each explant were determined.

Rooting of shoots

To stimulate root development, 1.5 to 2 cm long shoots were separated and grown in rooting medium under varying conditions:

- Medium 1: MS 1/2 with 30 g / L sucrose.
- Medium 2: MS 1/2 with 30 g / L sucrose and different IBA concentrations (0.1, 0.2, and 0.5 mg/L).
- Medium 3: same as medium 2 but with an initial incubation at 27 °C in the dark for 4 days before storing in the culture chamber.

The number of roots developed by the explants in each treatment was evaluated after 4 weeks.

Statistical analysis

Results are reported as mean±standard error. Differences between the treatments were analyzed using a t-test and one-way ANOVA with Duncan's post hoc test (p<0.05).

Results

Germination was first observed 3 d after incubation with an increase in both varieties until day 11. Following this period, germination of the Al-Hoceima variety was stable while the Tétouan variety showed slight increases. By the end of the germination period (day 35), 57% and 55% of Al-Hoceima and Tétouan varieties, respectively, had germinated (Figures 1 and 2).

We next evaluated the effects of BAP concentration (0.1, 0.5, 1, and 2 mg/L) on shoot growth from the germinated *O. ficus-indica* seeds (Figure 3, Tables 1 and 2).

The Al-Hoceima variety had the greatest number of buds per explant (12.280±0.667) at 0.5 mg/L BAP, while the longest buds (14.866±1.605 mm) were observed at 1.0 mg/L BAP. For the Tétouan variety, 0.5 mg/L BAP also generated the greatest number of buds (7.733±0.896); however, the longest buds (14.770±0.997 mm) were recorded at 0.1 mg/L BAP. In both varieties, increasing the BAP concentration to 2.0 mg/L decreased the number of buds (Figure 4).

The effects of BAP concentration on bud proliferation were then compared between the two *O. ficus-indica* varieties (Table 3). Across all BAP concentrations, the Al-Hoceima variety yielded more buds than the Tétouan variety with a significant difference at 0.5 mg/L BAP (p<0.001), at

which concentration both varieties produced the greatest number of buds. Tétouan variety bud length was significantly greater than Al-Hoceima at 0.1 mg/L BAP (p<0.006) but significantly shorter at 0.5 mg/L BAP (p<0.007), as Al-Hoceima bud length increased with BAP concentration up to 1.0 mg/L.

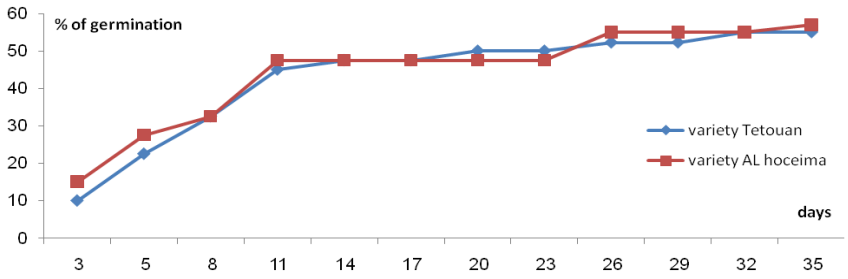

Figure 1. Progression of seed germination of *O. ficus-indica* varieties from Tétouan and Al-Hoceima.

Figure 2. *Opuntia ficus-indica* plants germinated in MS (Murashige and Skoog, 1962), Al-Hoceima variety.

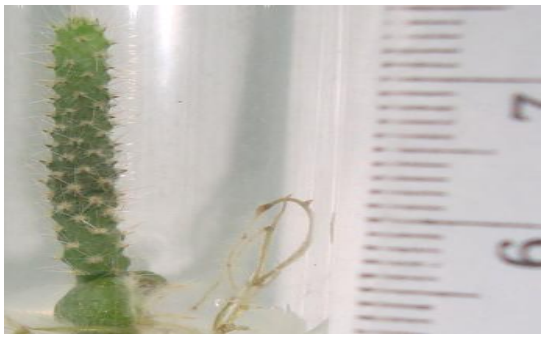

Figure 3. Seedlings of *Opuntia ficus-indica* in MS medium (Murashige and Skoog, 1962), Al-Hoceima variety which were cut into segments and used as a source of explants for multiplication.

Table 1. Effects of benzyladenine (BAP) concentration on Al-Hoceima *O. ficus-indica* bud proliferation *in vitro*.

Treatment (mg/L)	Length of buds (mm)	Number of buds
BAP0.1	10.5299^{b}±0.957896	6.440^{ab}±0.787570
BAP1.0	14.8667^{cd}±1.605300	7.840^{b}±0.684787
BAP0.5	12.6404^{bc}±0.805928	12.280^{c}±0.667133
BAP2.0	11.1858^{bc}±1.549209	6.200^{ab}±0.655744

Different letters within the same column indicate significant differences (p<0.05) by Duncan's post hoc test.

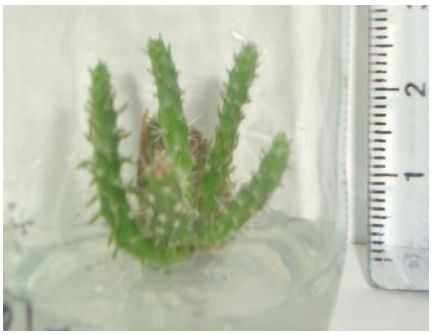

Figure 4. Multiple shoot proliferation on MS medium supplemented with benzyladenine (2 mg/L), Tétouan variety after 4 weeks.

Elongated shoots were transferred to different rooting supports to allow root formation. The effect of auxin supplementation on the number of roots was then investigated (Figure 5, Table 4). The Tétouan variety had significantly more roots in MS 1/2 30S+0.2 IBA medium (p=0.018) than the Al-Hoceima variety. When initially treated in the dark (first 4 d of cultivation), both varieties had a greater number of roots (Table 5). Interestingly, the Al-Hoceima variety cultivated in MS 1/2 30S+0.5IBA medium gave the greatest number of roots (15.05±0.630) and was significantly different from the Tétouan variety (p<0.001). Neither of the other media showed significant differences between the two varieties.

Table 2. Effects of benzyladenine (BAP) concentration on Tétouan *O. ficus-indica* bud proliferation *in vitro.*

Treatment (mg/L)	Length of buds (mm)	Number of buds
BAP0.1	14.7700^{c}±0.997280	5.93333^{ab}±0.917900
BAP1.0	11.2120^{b} ±1.190076	6.46667^{ab}±0.742369
BAP0.5	9.2570^{ab}±0.742840	7.73333^{b}±0.896908
BAP2.0	7.5660^{a}±0.457752	4.73333^{a}±0.520683

Different letters within the same column indicate significant differences ($p < 0.05$) by Duncan's post hoc test.

Table 3: Comparing the effects of benzyladenine (BAP; mg/L) on *in vitro* bud proliferation between *O. ficus-indica* varieties

N= 25	Origin	Length of buds (mm)	*p*-value t-test	Number of buds	*p*-value t-test
BAP0.1	Al-Hoceima	10.52992 ±0.957896	0.006	6.44000 ±0.787570	0.685
	Tétouan	14.77000 ±0.997280		5.93333 ±0.917900	
BAP0.5	Al-Hoceima	12.64040 ±0.805928	0.007	12.28000 ±0.667133	<0.001
	Tétouan	9.25733 ±0.742840		7.73333 ±0.896908	
BAP1.0	Al-Hoceima	14.86672 ±1.605300	0.116	7.84000 ±0.684787	0.201
	Tétouan	11.21200 ±1.190076		6.46667 ±0.742369	
BAP2.0	Al-Hoceima	11.18584 ±1.549209	0.084	6.20000 ±0.655744	0.127
	Tétouan	7.56600 ±0.457752		4.73333 ±0.520683	

Discussion

Cactus species can be propagated by different methods: seed, cuttings, or transplant (Santos-Díaz *et al.*, 2010). This work regenerated two varieties of *O. ficus-indica* from seeds collected from two provinces of northern Morocco (Tétouan and Al-Hoceima). Seeds were used to ensure the genetic diversity of the original populations was maintained. Following mechanical scarification of the seeds, maximal germination rates of 57% and 55% were achieved for Al-Hoceima and Tétouan varieties, respectively, which are comparable to results with mechanical scarification (Podda *et al.*, 2017), and more promising than those for two *Micranthocereus* species (Laila *et al.*, 2017). These germination rates suggest that *Opuntia* seeds have a dormancy stage and require a post-maturation period to reach a higher germination percentage, consistent with the germination behavior of other cactus species (Rojas-Aréchiga & Vázquez-Yanes, 2000; Montiel & Monta, 2003; Benítez-Rodríguez *et al.*, 2004; Esparza-Olguín *et al.*, 2005) .

Statistical analysis of the proliferation stage showed that BAP concentration differentially influences the shoot growth of the two varieties. Shoot growth environmental responses are dependent on genotype due to distinct nutritional requirements for optimal growth (Calderón-Paniagua1 *et al.*, 2001). In this study, supplementation with 0.5 mg/L BAP proved to be the most effective culture medium for optimal growth, similar to previous work (García-Saucedo *et al.*, 2005). Supplementation with 5.0 mg/L BAP has been reported to regenerate a large number of shoots by explant (Khalafalla *et al.*, 2007). However, we found that a high concentration of exogenous BAP decreased the number of buds produced by both *O. ficus-indica* varieties, in agreement with previous findings (Escobar A *et al.*, 1986).

Figure 5. Rooting of micropropagated shoots of *Opuntia ficus indica* Al-Hoceima variety.

In our study, BAP promoted the lengthening of regenerated shoots to a maximum value of 14.866±1.60 mm for the Al-Hoceima variety in 1.0 mg/L BAP. These results agree with previous work (Mohamed-Yasseen *et al.*, 1995) and suggest better efficacy than results

obtained with different cytokinins (El Finti *et al.*, 2012) and those using the *O. lanigera* Salm-Dyck species (Estrada-Luna *et al.*, 2008).

Table 4: Comparing the effects of indolebutyric acid (IBA) on the rooting of Tétouan and Al-Hoceima varieties of *O. ficus-indica* shoots after four weeks *in vitro* cultivation.

Treatment	Number of roots	*p*-value t-test
MS 1/2 30S_H	2.40000±0.255467	0.024
MS 1/2 30S_T	1.60000±0.190238	
MS 1/2 30S+0.1IBA_H	2.50000±0.235081	0.094
MS 1/2 30S+0.1IBA_T	3.46667±0.567926	
MS 1/2 30S+0.2IBA_H	1.65000±0.195677	0.018
MS 1/2 30S+0.2IBA_T	2.46667±0.273716	
MS 1/2 30S+0.5IBA_H	2.55000±0.276015	0.103
MS 1/2 30S+0.5IBA_T	3.40000±0.455652	

Values reported as mean±standard error. _H: Al-Hoceima (n=20). _T: Tétouan (n=15). MS 1/2 30S: Murashige and Skoog (MS) basal culture medium supplemented with 30 g/L sucrose. IBA in mg/L.

Table 5. Comparing the effects of indolebutyric acid (IBA) on the rooting of Tétouan and Al-Hoceima varieties of *O. ficus-indica* shoots after 4 weeks *in vitro* cultivation initiated in darkness (4 days).

Treatment+Darkness	Number of roots	*p*-value t-test
MS 1/2 30S+0.1IBA_Obs_H	8.65000±0.665997	0.101
MS 1/2 30S+0.1IBA_Obs _T	7.00000±0.696932	
MS 1/2 30S+0.2IBA_Obs _H	9.45000±0.642671	0.078
MS 1/2 30S+0.2IBA_Obs _T	7.66667±0.741085	
MS 1/2 30S+0.5IBA_Obs _H	15.05000±0.630267	**< 0.001**
MS 1/2 30S+0.5IBA_Obs _T	9.46667±1.086132	

Values reported as mean±standard error. _H: Al-Hoceima (n=20). _T: Tétouan (n=15). MS 1/2 30S: Murashige and Skoog (MS) basal culture medium supplemented with 30 g / L sucrose. IBA in mg/L.

The shoots generated in this study underwent rooting treatments with all shoots producing roots *in vitro* after four weeks in the presence or absence of exogenous auxin. Several *Opuntia* species have already been reported to root without auxins (Escobar A *et al.*, 1986; García-Saucedo *et al.*, 2005). In fact, applying IBA at various concentrations did not significantly increase the number of roots in our work. Fewer roots / explant were obtained in this study than have been reported under similar conditions (19.1 roots / explant; MS medium at half concentration supplemented with 0.5 mg/L IBA) (EL Finti *et al.*, 2013).

Initial treatment in darkness improves the number of roots / explant, reaching a maximum of 15.05 for the Al-Hoceima variety cultivated in MS 1/2 30S+0.5 IBA. This indicates that darkness promotes the formation of root meristems in agreement with a study examining the positive effect of temperature increases (from 25 °C to 30 °C) during dark treatment for the first week of rooting (Zimmerman, 1984).

Conclusion

These results describe an efficient *in vitro* proliferation system of shoots from seeds, providing a valuable tool for maintaining the genetic diversity of cacti. This protocol shows that cultivars of the same species have different responses to growth regulators during shoot proliferation and rooting.

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