MICROPROPAGATION OF CACTUS (*Opuntia ficus-indica*) AS STRATEGIC TOOL TO COMBAT DESERTIFICATION IN ARID AND SEMI ARID REGIONS

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ABSTRACT

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With aim of large production of plant material, a protocol for micropropagation of ***Opuntia ficus-indica*** was developed at the laboratory of plant tissue culture, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan, during the period of August 2006 to July 2007. Young cladode explant containing one areole was cultured on Murashige and Skoog medium (MS) supplemented with benzyladenine (BA) and kinetin (Kin) alone or in combination with naphthalene acetic acid (NAA). The highest shoot multiplication (26.5±1.74 shoots per explant) was achieved in 90 days on MS supplemented with BA (5.0 mg/l). Excised shoot cuttings of 3.0 cm were placed on the MS basal medium hormone-free or supplemented with different levels (0.25-1.0 mg/l) of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA. Although, satisfactory rooting percentage (100%) was achieved on both MS free-hormone or supplemented with IAA at 0.5 mg/l but, the highest number (15.0 ± 1.1) of root per shoot was obtained on MS medium supplemented with 0.5 mg/l IAA. Plants were successfully established in soil and adapted to greenhouse conditions. The protocols developed in this work provide a basis to achieve massive propagation of *O. ficus-indica* by *in vitro* culture of areoles as strategic tool to combat desertification in arid and semi arid regions.

Key words: Micropropagation, cactus, desertification, acclimatization.

INTRODUCTION

The Cactaceae family includes approximately 130 genera and 1500 species. Of these, the *Opuntia* and *Nopalea* genera are the most important due to their usefulness to man (Flores-Valdez and Osorio, 1996). *Opuntia* has a specialized photosynthetic mechanism known as Crassulacean Acid Metabolism (CAM), whereby these plants open their stomates and take up CO_2 at night, when temperatures are lower and humidity higher than during the daytime. This invariably results in reduced water loss (Nobel, 1995) offers exceptional possibilities for large quantities of biomass in water-limited areas that are useful for livestock feed (Felker *et al.* 2006).

Within the genus *Opuntia*, *Opuntia ficus-indica* is the most agronomically important species for the production of edible fruits and cladodes, which can be used as a vegetable and valuable forage resource in arid and semiarid lands during periods of drought and shortage of herbaceous plants (Scheinvar, 1995; Le Houérou, 2000; Juárez and Passera, 2002).

In Sudan, the arid and semiarid regions occupy around two-thirds of the total area (El Gamri, 2004); there fore the search for appropriate plant species to grow in this area is a permanent concern of most people living in harsh environments. *O. ficus-indica* represents the best choice since it shows great adaptability to various soil conditions, prevent environmental destruction caused by erosion. Moreover, farmers in many arid areas of the world use opuntia extensively as emergency forage that is harvested from both wild and cultivated populations to prevent the disastrous consequences of frequent and severe droughts (Le Houérou, 1992; Mirza, 2000; Medeiros *et al.* 2006). *Opuntia* has become an important crop for exotic fruit, vegetable, and forage production in Mexico, USA, Chile, Argentina, Israel, Italy, and South Africa, where it has adapted to dry areas with droughty conditions, scarce rainfall and poor, erosion prone soils (Pimienta-Barrios and Muñoz-Urias, 1995; Flores-Valdez, 1994).

Generally CAM plants are slow growing plants that sometimes have limited reproductive capacities and often have very specific and limited conditions for seed germination, flowering and seed production. Although, conventional propagation has been attempted for *Opuntia*, genetic segregation and slow growth and development represent serious practical problems (Malda *et al.* 1999). Moreover, cacti seeds are frequently difficult to be obtained (Mauseth, 1977) and plantlets are reported to be susceptible to damping-off (Mauseth, 1979; Ault and Blackmon, 1987). Therefore *in vitro* propagation by tissue culture is a feasible alternative option for the rapid multiplication and maintenance of germplasm (Smith *et al.* 1991; Johnson and Emino, 1979).Tissue culture has already proved to be successful for several members of the *Cactaceae* family (Johnson and Emino, 1979; Mauseth, 1979; Escobar *et al.* 1986; Clayton *et al.* 1990; Hubstenberger *et al.* 1992). However, for *O. ficus-indica* micropropagation only the effect of BA was tested (Garcıa- Saucedo., *et al.* 2005).

The main goal of this study was to achieve massive propagation of *O. ficus-indica* by *in vitro* culture of areoles, and to acclimatize propagated material to field conditions. The specific goals were to a) elaborate successful protocols

for cladode sterilization; b) find the optimal growth regulator and its concentration able to induce shooting from areoles of sterilized cladodes; c) determine optimum auxin concentrations able to induce rooting.

MATERIALS AND METHODS

Plant material

Young plants of *O. ficus-indica* were obtained from the cactus plantations of the Horticultural Department, Federal Ministry of Agriculture, Khartoum, Sudan. Plants were kept under green house conditions and used as a source for explants throughout the experiment.

Surface sterilization and isolation of explants

Young cactus cladodes were cut into pieces and surface sterilized by washing under running tap water and laundry bleach for 20 min. The pieces of the cladodes were sprayed with 70% ethanol, cleaned with a clean towel and transferred to a laminar flow. Under a laminar flow the cut pieces were immersed in 15% sodium hypochlorite for 20 min, and then rinsed in sterile distilled water for three times. Disinfested cladodes were cut to 1.0 cm^2 pieces each containing one areole and transferred into Petri dishes as explant ready for inoculation.

Inoculation

Explants were cultured in culture bottles containing MS (Murashige and Skoog, 1962) basal media supplemented with benzyladenine (BA) (0.5, 1.0, 1.5, 3.0 and 5.0 mg L^{-1}) or Kinetin (Kin) (0.5, 1.0, 1.5, 3.0 and 5.0 mg L^{-1}) alone or in combination with naphthaleneacetic acid (NAA) (0.5 mg L^{-1}). All media were prepared by standard procedures and the culture bottles were transferred to growth chamber with a photoperiod of 16 h light and 8 h of dark at 25ºC ± 2 ºC and incubated for 3 months.

Rooting of in vitro induced shoots

Shoots (3.0 cm) derived from shoot bunches were excised and rooted on medium consisting of MS basal medium supplemented with NAA, Indole Acetic Acid (IAA) or Indole Butric Acid (IBA) each at 0.25, 0.5 or 1.0 mg/l. All the media used in this study were supplemented with 3% (w/v) sucrose, solidified with 0.8% (w/v) agar and the pH was adjusted to 5.8± 0.1 before autoclaving at 121°C and 15 lb psi for 15 min.

Acclimatization

In vitro rooted plants were removed from rooting medium and washed to remove adhering gel and transplanted to plastic pots containing autoclaved garden soil and sand at 3:1 ratio. Plants were kept under culture room conditions for 15 days then transferred to green house and placed under shade until growth was observed.

Statistical analysis

Results on the number of buds and shoot per explants, rooting percentage and the number of roots developed per shoot in each treatment was observed at regular intervals. Data were collected from three independent experiments and subjected to analysis of variance. Means were compared with Duncan's Multiple Range Test (Duncan, 1955) and presented as average ± standard error (SE).

RESULTS AND DISCUSSION

The surface sterilization of cacti is extremely important because the thorns and hairs normally found in such plants host a large variety of microorganisms (Garcıa- Saucedo., *et al.* 2005) Here in this study, washing the cladodes of the *O. ficus-indica* under running tap water and laundry bleach followed by the standard method of ethanol/ sodium hypochlorite was insufficient for the *in vitro* establishment of this species. The surface sterilization was only efficient when the pieces of the cladodes after washing under running tap water and laundry bleach for 20 min were sprayed with 70% ethanol, cleaned with a clean towel and transferred to a laminar flow and immersed in 15% sodium hypochlorite for 20 min then rinsed in sterile distilled water for three times

The morphogenetic responses of cladodes explants (Figure 1A) to BA and Kin either alone or in combination with NAA are summarized in Table 1. Explants cultured on MS medium without growth regulator did not show any response. However on MS medium supplemented with growth regulators a 100% of all areole swell in their size and a varying degree of shoot bud differentiation was observed under a 16-h photoperiod after one months of culture. This is consistent with results of Juarezi and Passera, 2002, who found that 100% areole shooting, was obtained on solid medium containing BA, under a 16-h photoperiod on the 35th day of culture.

Table 1. Effects of benzyladenine (BA) and kinetin (Kin) alone or in combination with 1-naphtalenacetic acid (NAA) on different morphogenetic responses of *Opuntia ficus-indica* on MS medium after3 months of culture

Growth regulator (mg/l)			Bud forming explant (%)	Number of bud per explant (Mean± SE)	Number of shoot per explant (Mean± SE)
BA	Kin	NAA			
0.5	0.0	0.0	100	22.8 ± 2.4 ^{j}	3.0 ± 0.26 ^{cd}
1.0	0.0	0.0	100	31.8 ± 2.9 ^{hi}	4.5 ± 0.51 ^{cd}
1.5	0.0	0.0	100	53.0 ± 5.7 ^{de}	5.3 ± 0.43 ^{cd}
3.0	0.0	0.0	100	52.5 ± 8.6 ^{de}	7.0 ± 0.53 ^{cd}
5.0	0.0	0.0	100	31.8 ± 2.0 ^{hi}	26.5 ± 1.74 ^{a}
0.0	0.5	0.0	100	72.5 ± 6.6 ^{c}	5.5 ± 0.44 ^{cd}
0.0	1.0	0.0	100	49.8 ± 9.3 ^{e}	7.3 ± 0.50 ^{cd}
0.0	1.5	0.0	100	40.5 ± 1.7 ^{fg}	8.0 ± 0.53 ^{c}
0.0	3.0	0.0	100	33.5 ± 4.1 ^{ghi}	3.5 ± 0.23 ^{cd}
0.0	5.0	0.0	100	30.0 ± 1.8 ^{i}	2.7 ± 0.34 ^{d}
0.5	0.0	0.5	100	31.5 ± 3.9 ^{hi}	4.0 ± 0.37 ^{cd}
1.0	0.0	0.5	100	30.5 ± 2.7^{i}	4.5 ± 0.03 ^{cd}
3.0	0.0	0.5	100	46.3 ± 3.4 ^{ef}	10.2 ± 0.72 ^{bc}
5.0	0.0	0.5	100	58.7 ± 5.2 ^{d}	17.8 ± 2.07 ^{b}
0.0	0.5	0.5	100	47.5 ± 10.9 ^{ef}	7.0 ± 0.70 ^{cd}
0.0	1.0	0.5	100	39.8 ± 5.8 ^{fgh}	6.5 ± 0.78 ^{cd}
0.0	3.0	0.5	100	93.3 ± 15.6 ^{b}	3.8 ± 0.49 ^{cd}
0.0	5.0	0.5	100	104.7 ± 12.6 ^{a}	2.0 ± 0.26 ^{d}

Means with same letter (s) in the same column are not significantly different at 5% using Duncan's multiple range test

All the concentrations of BA and Kin alone or in combination with NAA facilitate bud and shoot differentiation, but after three months of culture BA alone being the most efficient in terms of number of shoot per explant. Shoots number increases with the increase of BA concentration. BA at the highest concentration (5.0 mg/l) gave the highest number of shoot per explant (26.5±1.74) (Figure 1B), thus being the most efficient growth regulator tested for the optimal multiplication of this plant material. Generally cytokinin is considered to be essential for the development of cactus axillary shoots (Mauseth, 1977). Mohamed-Yassen (1995) reported that, increasing the BA concentration to 8.8 µM resulted in significantly more shoots being produced. Moreover, the effectiveness of BA in shoot differentiation has been documented in number of cactus plants such as *Opuntia amyclea* (Escobar *et al*.1986).

The synergistic influence of NAA with cytokinins (BA and Kin) did not improve the number of shoots per explant. However, Kin in combination with NAA markedly enhanced the number of buds per explant. Kin at 5.0 mg/l in combination with 0.5 mg/l NAA induced the maximum number of buds per explant (104.7 ± 12.6).However, the induced buds failed to elongate on the same medium resulting in rosettes of shoots compared to those obtained on media containing BA a lone (Figure 2). These findings are in agreement with those reported by Bustamante and Heras (1990) on Cacti (*Pelecyphora aselliformis*) and *Nealolydia lophophoroides*; Feng-Feng *et al*. (2000) on *Aloe barbebsis* and Mata-Rosas (2001) on *Turbinicapus laui*. They concluded that applying a combination of BA and NAA in different concentrations was a limiting factor for shoot formation. Moreover, Clayton *et al*., 1990 reported that low levels of auxin with cytokinin increased axillary bud production in some cactus species. Previous studies have pointed out that the optimal hormone combination may be unique for each cactus species (Johnson and Emino, 1977, 1979).

Elongated and well-developed (3.0 cm) shoots derived from cladodes explants (grown on MS medium supplemented with 5.0 mg/L BA) were excised from the shoot clumps and transferred to MS medium hormone-free or augmented with 0.25, 0.5 or 1.0 mg/l of either IAA, NAA or IBA for root initiation. Of the three auxins tested, IAA at 0.5 induced the maximum rooting percentage (100%) and the highest number (15.0 ± 1.1) of roots per rooted plantlet (Table 2) (Figure 1c) after two weeks of culture. The use of MS medium for rooting of *in vitro* induced shoots has already been reported for *O. ficus indica* (Garcıa- Saucedo *et al*. 2005). Here in this study 100% rooting percentage

and 4.0 ± 0.5 roots per shoot were achieved on MS free-hormone media. Similar to our result *Opuntia amyclaea* was reported to have rooted on medium without the supply of exogenous auxin (Escobar *et al*. 1986). According to Clayton *et al*. (1990), many cacti produce an excess of auxin under *in vitro* culture conditions, hence facilitate *in vitro* rooting in MS hormone -free.

Table 2. The effect of auxins on rooting of in vitro – derived shoots of opuntia *puntiaficus-indica*) after two weeks of culture on MS medium

Auxins (mg/l)			Rooting (%)	Number of roots per shoot (Mean ±SE)
NAA	IBA	IAA		
0.0	0.0	0.0	100.0	4.0 ± 0.5^{e}
0.25	0.0	0.0	83.3	5.5 ± 0.6^{de}
0.50	0.0	0.0	66.0	7.3 ± 0.7^{de}
1.0	0.0	0.0	66.0	12.3 ± 1.7^{ab}
0.0	0.25	0.0	66.0	8.7 ± 0.9^{cd}
0.0	0.50	0.0	50.0	9.0 ± 0.7^{bcd}
0.0	1.0	0.0	50.0	8.0 ± 0.6^{cd}
0.0	0.0	0.25	83.3	11.7 ± 0.9^{abc}
0.0	0.0	0.50	100.0	15.0 ± 1.1^{a}
0.0	0.0	1.0	66.0	13.7 ± 0.8^{a}

Means with same letter (s) in the same column are not significantly different at 5% using Duncan's multiple range test.

The success of any *in vitro* regeneration protocol largely depends on the survival and growth performance of propagated plantlets *ex vitro* (Joshi and Dhar, 2003).In the present study, the acclimatization procedures applied was successful (Figure 1D). *In vitro* regenerated plantlets showed 100% survival when transferred to soil and there was no detectable variation among the plants with respect to morphology and growth characteristics (Figure 1E). Similar result was shown by Malda *et al*. 1999, who reported that, although water loss was relatively significant during acclimatization, survival of cacti was not affected, suggesting that body succulence allowed plants to survive and recover after a certain degree of desiccation.

In conclusion, *O. ficus-indica* micro propagation cycle based on proliferation of vegetative tissues includes establishment of tissue culture through *in vitro* introduction of shoot meristem with reduced fungal and bacterial contamination, meristem and shoot proliferation, root induction and plant acclimatization summarized in 5 steps (Figure 3). The complete cycle from introduction of cladodes into tissue culture to recovery of greenhouse grown plants requires about 6 months. By this means it was possible to generate large numbers of shoots in one cycle, which proves useful for the establishment of a continuous plant production system. The establishment of *in vitro* tissue culture of *O. ficus-indica* did not show serious difficulties, except for the surface sterilization. The results of this study suggest that *O. ficus-indica* can be successfully micropropagated by areoles, and easily acclimatized to field conditions hence represent the best strategic tool to combat desertification in arid and semi arid regions of Sudan.

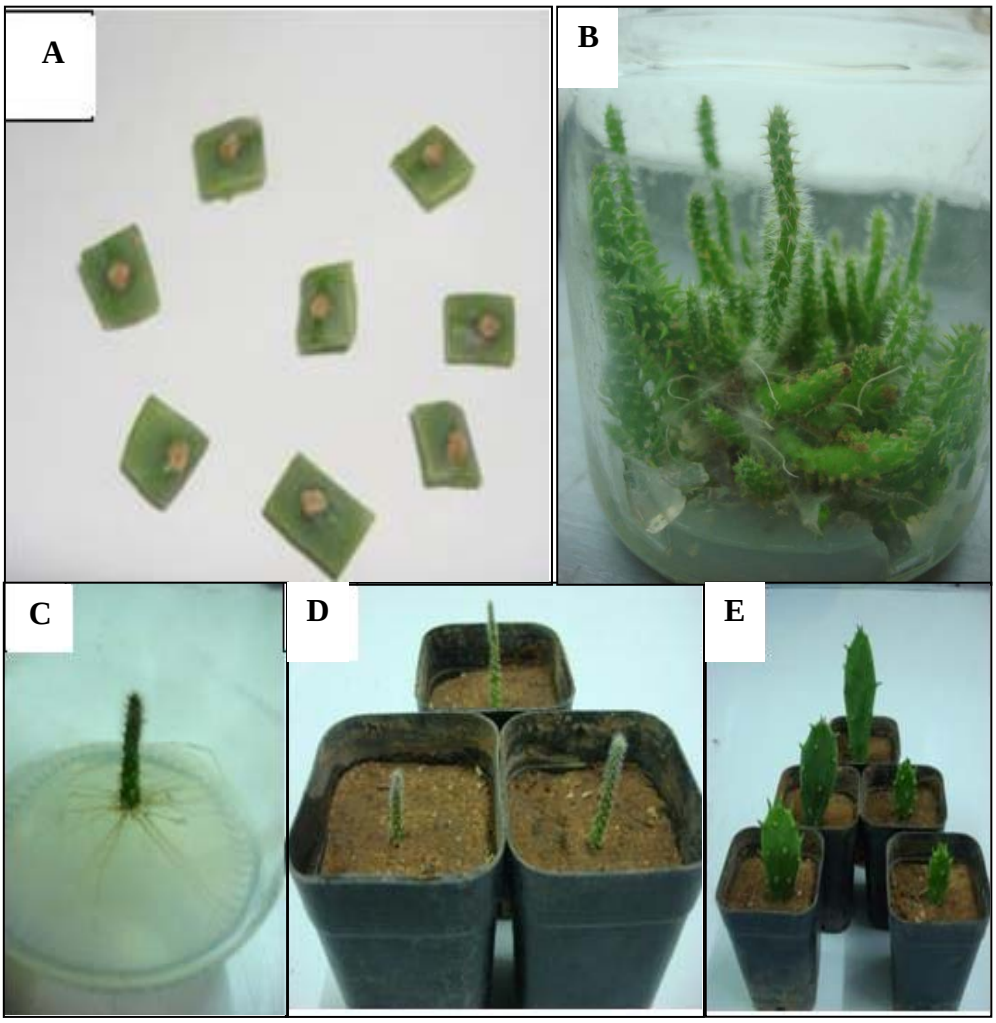

Figure 1. *In vitro* micropropagation system for cactus (*Opuntia ficus-indica*) (A-E). A. Cladode explants containing one areole. B, Multiple shoot formation on MS medium supplemented with BA (5.0 mg/l). C, Root induction on MS supplemented with IAA at 0.5 mg/l. D, Plant acclimatization in growth chamber. E, Recovered plant in greenhouse

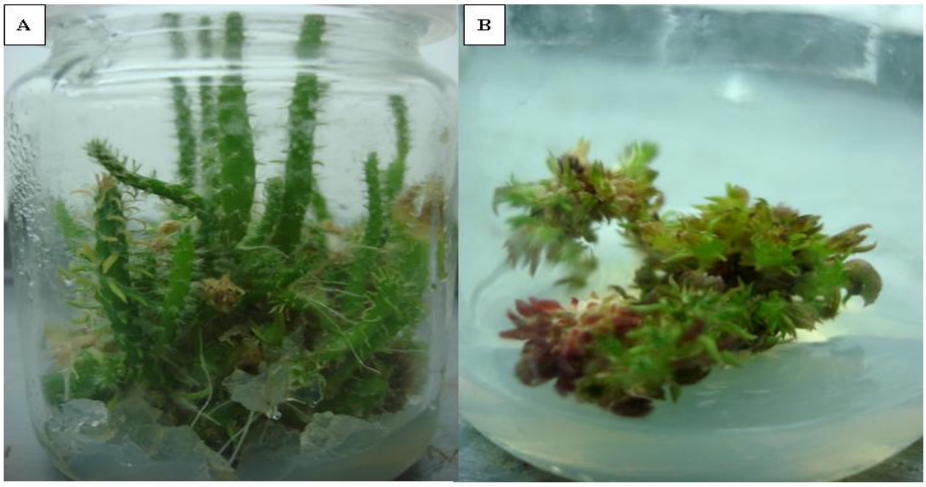

Figure 2. Influence of growth regulator on cactus (*Opuntia ficus-indica*) shoots bud differentiation. A. Multiple shoot proliferation on MS medium supplemented with benzyladenine. B. Rosettes of shoots induced on MS medium supplemented with kinetin in combination with NAA

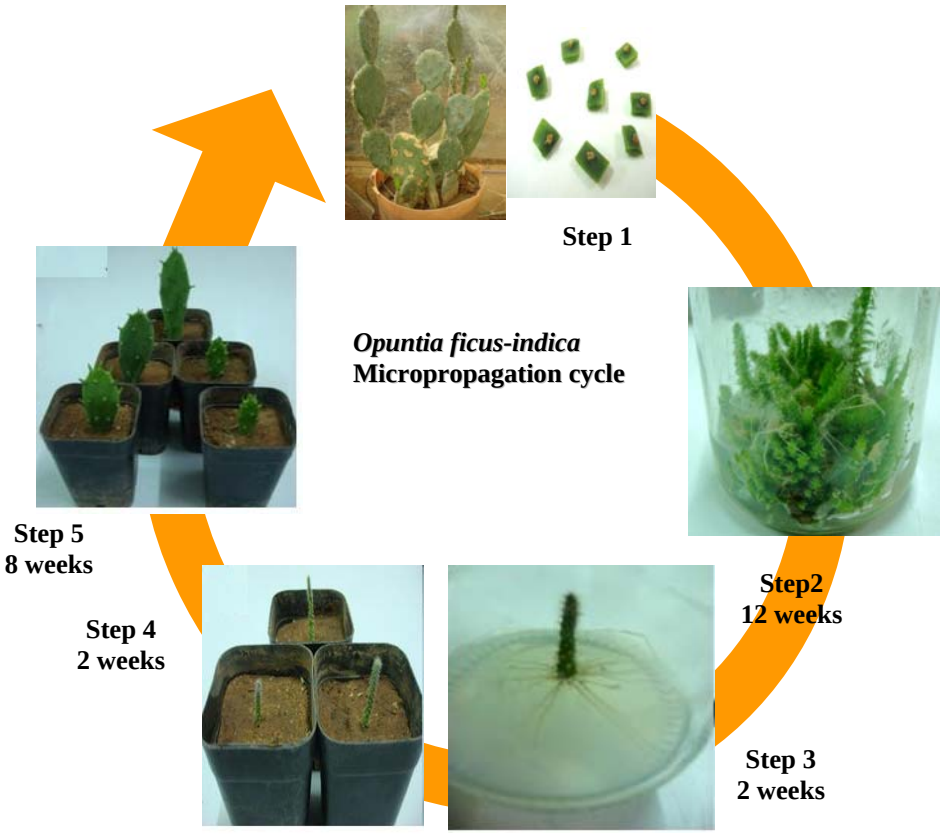

Figure 3. Six-month opuntia (*Opuntia ficus-indica*) micropropagation cycle

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