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Short Communication

Influence of Cytokinin and Auxin Types and Concentrations on *In Vitro* Shoot and Root Regeneration of Cactus Pear [(*Opuntia ficus-indica* (L.) Mill.]

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Abstract

An experiment was conducted with the aim of developing a protocol for the *in vitro* propagation of cactus pear from *in vitro* derived areole explants. The protocol involves subsequent *in vitro* morphogenesis and rooting of the *in vitro* proliferated shoots. For the shoot proliferation stage, explants each with 1 to 2 areoles were cultured on Murashige and Skoog medium (MS) supplemented with benzyladenine (BA) and kinetin (Kin) each with concentrations of 0, 0.5, 1.0, 2.0 and 4 mg/l and two combinations (0.5 mg/l BA + 4.0 mg/l Kin and 0.5 mg/l Kin + 4.0 mg/l BA). Analysis of the results indicated that there was no significant difference among the treatments used in terms of percentage of bud formation. Kinetin at 2.0, 1.0 and 0.5 mg/l were the best for days to shoot emergence. The highest bud numbers (95.75 buds per explant) were achieved on medium supplemented with Kin at 1.0 and 2.0 mg/l. Culturing the explants on MS medium amended with Kin at 1.0 mg/l concentration significantly augmented the shootlet proliferation rates to 29.5-fold higher than on MS-free medium. The best result for shoot length (4.7 cm) was obtained from medium with 0.5 mg/l Kin. Similarly, Kin at 0.5, 1.0 mg/l and BA at 2.0 mg/l yielded maximum plant mass production (shoot fresh and dry weight). Elongated shoot cuttings were placed on the medium (0, 0.25, 0.5, 1.0 and 1.5 mg/l) of naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) and (0.25 mg/l IBA + 1.5 mg/l NAA and 1.5 mg/l IBA + 0.25 mg/l NAA) for *in vitro* root induction. Hundred percent rooting was achieved on all of the treatments tested in two weeks of culture except the PGR free medium. The most efficient growth treatment for the longest root length (10.75 cm), maximum root fresh weight (1.88 gm) and root dry weight (0.41 gm) was IBA at 1.5 mg/l. However, there was no significance difference between IBA at 1.0 mg/l and IBA at 1.5 mg/l regarding to root length per explant. The best result for root number (10.0 roots per explant) was obtained from medium supplemented with 1.5 mg/l NAA. In conclusion, it is beneficial to use the *in vitro* protocol developed in this study for mass micropropagation and circumvent the challenges of conventional *in vivo* multiplication of cactus pear.

Key words: Areole, BA, Cactus pear, Ethiopia, IBA, *In vitro* propagation, Kin, MS-medium, NAA

Introduction

The cactus pear belongs to the Cactaceae family and it is a species originated on the plateau of southern and central Mexico (Griffith, 2004). It is extensively used as food for humans and feed for animals. This plant has been domesticated as an important fruit crop in many areas of the world although wild populations are still unexploited (Mondragon, 2001). The cactus pear is cultivated in more than 30 countries of the world including Ethiopia primarily for its fruit, but also the young cladodes are used as vegetable and mature ones as cattle feed. It has become an important crop for exotic fruit, vegetable, and forage production, where it has adapted to dry areas with poor and erosion prone soils (Flores-Valdez, 1992; Pimienta-Barrios and Muñoz, 1995).

Agriculture in northern Ethiopia, like in other parts of the country, is the main sector on which more than 85% of the population is directly dependent on it. Northern Ethiopia is characterized by long periods of drought and unreliable rainfall, compounded by excessive human and livestock pressures on the land resulting in high food insecurity. It is in this context that the cactus pear plays an increasingly vital role as a source of food and feed. This plant is also used as a fuel, as a live fence or hedge, for soil conservation and its fresh fruit as a limited source of household income. In this regard, cactus has been an integral and impressive part of the culture and economy of Tigray. However due to low attention, this ample cactus pear resource of the region is still underutilized (Fesseha, 2010).

In spite of their economic importance, few attempts have been made to improve the characteristics of cactus cultivars (Mohamed-Yassen *et al*., 1995). Although, seed propagation has been attempted for *Opuntia*, a continuous genetic segregation due to cross pollination or natural hybridization occurs and as a result seedlings are genetically and phenotypically non-uniform. In addition, cacti species have long juvenile stage and these represent serious practical problems for propagation and multiplication (Malda *et al.*, 1999). Moreover, cacti seeds are frequently difficult to be obtained and plantlets are reported to be susceptible to damping-off, golden spot and black soft rots (Mauseth, 1979; Ault and Blackmon, 1987). In addition, cacti and other plants which possess CAM photosynthetic pathway usually present limited reproductive capacities and often have very specific and limited conditions for seed germination, flowering and seed production. Thus, most propagation for *Opuntia* is clonal, and it is done vegetatively through cladodes (stem cuttings) (Escobar-Araya *et al*., 1986; Llamoca *et al*., 1999). This method of propagation is easy to perform and efficient; however, their propagation

rates are relatively low and require large spaces for propagation. Though the conventional propagation techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large-scale cultivation in a short period by rapid mass multiplication. Therefore, *in vitro* propagation by tissue culture is the most promising and feasible alternative option because it provides rapid regeneration and multiplication rates, efficient germplasm maintenance, reduced requirements for space, the production of healthy and pathogen-free cacti plants (Johnson and Emino, 1979; Smith *et al.*, 1991). Hence, for the successful use of plant tissue culture technologies for crop improvement, an efficient and reproducible regeneration procedure is a pre-requisite.

Thus, the aim of this study was to determine the optimal cytokinin and auxin types and concentrations to induce shooting and rooting from areoles of sterilized cladodes.

Materials and Methods

Selection and preparation of explants

Explants were obtained from the mother plants maintained at the cactus nursery site of Mekelle Agricultural Research Center. These plant materials were collections of three major cactus growing areas of the region (Adigrat, Erob and Mehoni) The genotype used in this study was selected by the growers and as it was the most commonly cultivated one in the region. To start the process of micropropagation, healthy young cladodes with no visual signs of pathogenic infections, each with active areoles, were selected from donor plants. The cladodes were carefully excised just at the base using a sterile surgical blade and transported to the Plant Biotechnology Laboratory with a well disinfected plastic bag for further sterilization.

Sterilization and inoculation of explants

Spines were carefully removed with forceps and trimmed with regular scissors prior to disinfection and detachment from mother cladode. Entire cladodes were gently washed under running tap water several times, soaked in a solution containing commercial detergent (Liqui-Nox, Alconox Inc., New York) for 20 min and then rinsed three times in distilled water. The cladodes were sprayed with 70% ethanol and cleaned with a clean towel and then rinsed in distilled water for three times. They were again cut into small pieces, each containing 1 to 2 areoles (Figure 1). The pieces of the plant tissue were kept as large as possible for further surface sterilization procedure in order to minimize damage by the sterilant. Cut pieces were disinfected by immersing in a kocide solution for 2 hr followed by rinsing in sterile distilled water. Treated tissues were then immersed in commercial Clorox (NaOCl) (5%) with

0.1% Tween-80 as surfactant for 20 min followed by rinsing five times with sterile distilled water and transferred to a laminar air flow. Under the laminar hood, explants were immersed in 70% ethanol for 1 min followed by three rinses with sterile water (Figure 2).

The surface disinfected explants were cut to $1cm^2$ pieces. Subsequently the explants were washed gently with sterile distilled water in aseptic condition under the laminar flow hood. Again explants were immersed in 5% NaOCl for 15 min and then thoroughly washed five times with sterile distilled water. Finally the explants were trimmed to remove the chlorine-damaged areas using a sterilized blades and forceps.The surface sterilized and trimmed explants were cultured in a MS (Murashige and Skoog, 1962) medium to induce areole activation and shoot growth. Equipments like forceps, blades and other working materials were sterilized using a Glass Bid Sterilizer under the laminar flow hood during inoculation. After inoculation, each culture vessel was sealed with a parafilm. Then the vessels were transferred into the growth room and kept under 16:8 h (light: dark) photoperiod regime with light intensity of 2000-2500 lux, temperature of 25 ± 2°C and 70% relative humidity (RH).

Experiment set up and culture conditions

Shoots obtained in the induction phase were used as source of secondary explants for propagation experiments. The explants were aseptically cultured on full strength MS medium supplemented with benzyl adenine (BA) (0.5, 1.0, 2.0, and 4.0 mg/l), kinetin (Kin) (0.5, 1.0, 2.0, and 4.0 mg/l), 0.5 mg/l BA + 4.0 mg/l Kin and 4.0 mg/l BA + 0.5 mg/l Kin and MS medium (control). The control consisted of cultures without cytokinins. Four explants were cultured on 40 ml of medium contained in each culture vessel, with a total replicate (observation unit) of sixteen explants per treatment. All media were prepared by standard procedures and the pH was adjusted to 5.8 with 0.1% N HCl prior to autoclaving at 121^0C for 20 min. Culture were maintained at 25^0C ± 2^0C in the growth room under a 16 h light/8 h dark photoperiod regime and incubated for 3 months. A constant photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} was provided by cool white fluorescent light. Data on bud forming explants (%), number of days to shoot emergence, number of buds per explant, shoot number per explant, shoot height, shoot fresh weight and dry weight were recorded after 4, 8 and 12 weeks in culture. The combined data were used for statistical analysis.

Shoots derived from shoot bunches were excised and rooted on medium consisting of MS basal medium supplemented with Naphtalee acetic acid (NAA) and Indole Butric Acid (IBA) each at 0.25, 0.5, 1.0 and 1.5 mg/l, and 0.25 mg/l IBA + 1.5 mg/l NAA, 1.5 mg/l IBA + 0.25 mg/l NAA and MS-

hormone free (control) . Sucrose (3%) was used as carbon source and media were solidified with agar-agar (0.8%). The pH was adjusted to 5.8 with 0.1% N NaOH prior to autoclaving at 121^{0}C and 102 kpa for 20 min. The cultures were completely randomized and maintained in a growth room at 25 ± 2^{0}C under a 16 h photoperiod. After two weeks of culture, the number of roots produced per shoot, root length, shoot length, root fresh weight, root dry weight and percentage of cultured shoots producing roots were recorded from each treatment.

Statistical Analysis

Treatment effects in all experiments were determined by using the analysis of variance (ANOVA) and significant differences among treatments were determined by Duncan's Multiple Range Test using the SAS (version 9.0) software package.

Results and Discussion

During the initiation phase, contamination of cultures was a serious problem since explants were directly brought from the field grown plant materials. However, the disinfection was efficient when the spines were carefully removed with forceps or trimmed with regular scissors prior to disinfection and detachment from mother cladode and then surface sterilization at ambient condition followed by sterilization in a laminar flow hood. Since these spines harbor several microorganisms, the effectiveness of the sterilant will be high when we remove the spines from the cladode. This particular manipulation of donor plants and young cladodes plus the effectiveness of the disinfection protocol used resulted in free-pathogen cultures (0% contamination), which is of especial importance since bacteria and fungi contamination is common and usually cause serious problems in most cacti tissue culture protocols. For the proliferation stage, the ANOVA detected significant effects on type and concentrations of cytokinin. The analysis of variance revealed that the different cytokinin concentrations of BAP and Kin tested had a highly significant ($p<0.01$) effects on the number of days to shoot emergence, bud number, shoot number, shoot length, shoot fresh weight as well as shoot dry weight of cactus *in vitro* (Table 1).

The morphogenetic responses of explants to BA and Kin are summarized in Table 1. A 100 % bud formation was observed under a 16-h photoperiod after one month of incubation period. This is consistent with results of Khalafalla *et al*. (2007), who found that a 100% bud formation on solid medium containing benzyladenine (BA) and kinetin (Kin) alone or in combination with 1-naphtalenacetic acid (NAA) under a 16-h photoperiod after 30 days of culture. Their study has shown that the different concentrations of cytokinin hormones used in the investigation had similar effect on bud forming ability. Llamoca *et al*. (1999) reported best bud formation in *O. ficus-indica*

Cultivar Gigante with the application of 2.2 µM BA with or without GA_3.

Explants cultured on medium supplemented with Kin at 2.0, 1.0, and 0.5 mg/l have proliferated shoots quickly; *i.e.*, 9.25, 10.5 and 11.25 days, respectively; though they were statistically non-significant for the average number of days to shoot emergence (Table 1). On the other hand, explants cultured on medium supplemented with no hormone were late (15.0) in days to shoot emergence followed by BA at 0.5, 1.0 and 4.0 mg/l, 0.5 mg/l BA + 4 mg/l Kin and on 4.0 mg/l BA + 0.5 mg/l Kin (Table 1). There were no statistical differences among these treatments and the control group. Thus, it is economical to use 0.5 mg/l Kin instead of the other concentration levels in order to get a fast induction of shoots in cactus tissue culture.

Table 1. Effects of different concentrations of BA and Kin on the different morphogenetic responses of cactus pear after three months of culturing.

Cytokinins (mg/l)		Bud forming explant (%)	Number of days to shoot emergence (n)	Number of buds per explant (n)	Number of shoots per explant (n)	Shoot length per explant (cm)	Shoot fresh weight per explants (g)	Shoot dry weight per explant (g)
BA	Kin							
0.0	0.0	100	15.00a	16.50g	1.00h	1.83h	0.733g	0.041g
0.5	0.0	100	14.25a	23.00f	4.25fg	2.80ef	3.068e	0.162e
1.0	0.0	100	14.25a	36.25e	10.75d	3.03cde	4.780b	0.203c
2.0	0.0	100	11.75bc	57.50d	24.75b	3.10cd	5.645a	0.241b
4.0	0.0	100	13.25ab	23.75f	4.00g	2.13g	2.455f	0.143f
0.0	0.5	100	11.25cd	84.00b	15.50c	4.70a	4.975a	0.240b
0.0	1.0	100	10.50cd	95.75a	29.50a	3.88b	5.775a	0.279a
0.0	2.0	100	9.25d	95.75a	25.25b	3.23c	4.098c	0.195cd
0.0	4.0	100	12.00bc	63.00c	11.75d	2.75f	3.305e	0.163e
0.5	4.0	100	14.25a	26.63f	8.60e	2.85def	3.325d	0.183d
4.0	0.5	100	13.25ab	22.38f	6.13f	2.25g	2.460f	0.153ef
CV (%)		0.00	9.69	5.58	10.70	5.88	6.130	6.170

Means with same letter (s) in the same column are not significantly different according to Duncan's Multiple Range Test (α = 0.01).

Regarding bud number, the concentrations of Kin at 1.0 and 2.0 mg/l produced a balanced response with the highest number of activated buds (95.75) that generated shoots with a bigger size than the rest of treatments (Table 1). However, the mean minimum number of buds (16.5) per explant was attained on MS hormone-free medium. For some reason, the synergetic influence of BA with Kin did not improve the number of buds and shoots per explant. We observed that some explants cultured in these treatments have slow growth rate, do not respond to the subculture or die after several days. It seems that the responses are particular or depend on the type and concentration of cytokinin, the target tissue and some other factors. In general, when the concentration of BA was increased from 1.0 to 4.0 mg/l in the medium, the mean bud number per explant was markedly enhanced upto 2 mg/l concentration level and declined

afterwards (Table 1). According to Khalafalla *et al*. (2007), Kin at 0.5 and 1.0 mg/l and BA at 1.5 and 3.0 mg/l induced 72.5 ± 6.6, 49.8 ± 9.3, 53.0 ± 5.7 and 52.5 ± 8.6 buds per explants, respectively.

As can be seen from Table 1, the best response for shoot number was obtained from the explants cultured on the medium supplemented with 1.0 mg/l Kin followed by Kin at 2.0 mg/l and BA at 2.0 mg/l (Figure 3A and B). No significant difference was observed interms of shoot number between Kin and BA at 2.0 mg/l. It was observed that reculturing the culture of shootlet explants for three times at monthly intervals greatly increased the cumulative number of the formed shootlets. The maximum number of shootlets obtained from 1.0 mg/l Kin represents 29.5-fold higher compared to the explants cultured on hormone-free medium (Table 1). In addition, the shoots obtained on this cytokinin concentration showed morphologically good-looking, well differentiated, relatively taller and deep green along with high shoot growth rate plantlets. These results showed that the most adequate culture medium for efficient shoot proliferation were MS-medium supplemented with Kin 1 mg/l (29.5) followed by Kin at 2 mg/l (25.25) and BA at 2 mg/l (24.75). Thus, Kin hormone is the most efficient growth regulator tested for the optimal multiplication of the plant material in this study. The least number of shoots per explant (1.0) was observed in hormone-free medium (Table 1). In *Opuntia amyclaea,* a low concentration (2.25 mg/l) of BA produced one of the best results obtained for cacti averaging the development of 15 shoots per explant (Escobar-Araya *et al*., 1986). Generally, cytokinin is considered to be essential for the development of cactus axillary shoots (Mauseth, 1977). In contrary to our study, Khalafalla *et al*. (2007) reported that BA at the highest concentration (5.0 mg/l) gave the highest number of shoot per explant and they concluded that shoot number increased with the increase of BA concentration. Similarly, Sawsan *et al*. (2005) concluded that the highest number of shootlets (47.3) obtained on cactus (*Cereus peruvianus* L.) was from explants cultured on medium provided with BAP (5 mg/l) plus NAA (0.1 mg/l). However, in this present study shoot number reduced from 24.75 to 4 as BA concentration increased from 2mg to 4mg.

The medium supplemented with 0.5 mg/l Kin produced the greatest mean shoot length per explant (4.7 cm) followed by 1 mg/l Kin (3.88 cm) (Table 1). These results were significantly higher in comparison to data obtained from treatments supplemented with BA. In contrast with this observation, the minimum length of shoot per explant (1.83 cm) was recorded on medium supplemented with no growth hormone (control). Although not consistent throughout, the mean number of shootlets and mean shoot length per explant were generally found to have declined with the

increase in the concentration of Kin levels from 0.0 to 4 mg/l (Table 1). These results were similar with those reported by Garcia *et al.* (2005) who also observed better bud formation and shoot elongation in *opuntia* species at lower BA concentration than at higher concentration. In general, BA produced shoots of smaller size as compared to treatments consisting of Kin that produced shoots with higher size.

In this study, mean shoot fresh weight and dry weight among the different cytokinin concentrations were found to be highly significantly ($p<0.01$) different. The maximum shoot fresh weight (5.775 g) as well as shoot dry weight per explant (0.279 g) was recorded on MS medium fortified with Kin 1.0 mg/l (Table 1). The average shoot fresh weight obtained from this treatment is statistically non-significant from those recorded in 2.0 mg/l BA (5.645 gm) and in 0.5 mg/l Kin (4.975 g). In contrast, the minimum shoot fresh weight (0.733 g) and shoot dry weight (0.041 g) per explant was recorded on MS medium with no cytokinin. In general, an inverse relationship was observed between kin concentration and biomass weight.

In most cases of the shoot proliferation stage, Kin at most of the concentration levels was comparatively more effective in inducing more shoots as compared to BA. As a general observation, the explants exhibited reduced shoot induction that failed to proliferate at the highest levels of both cytokinin concentrations, thereby resulting in poor performance of most shoot growth and development characters tested in this experiment (Table 1).

In Vitro rooting of regenerated shoots

The analysis of variance revealed that all the examined auxin concentrations had highly significant ($p<0.01$) effects on rooting response, root number, root length, shoot length, root fresh weight and root dry weight of *in vitro* derived shoots of cactus pear plantlets.

Data presented in Table 2 revealed that the entire MS basal medium supplemented with different concentrations of IBA and NAA induced roots in two weeks after culturing. Root formation was not observed when shoots were cultured on a medium lacking auxin. However, after 35 days of culturing, some explants have started rooting on MS medium without the supply of exogenous auxins (data not presented).

The number of main roots formed per shootlet explant was significantly affected by the different types as well as concentrations of the auxins used in this study. The highest number of main roots per explant (10.0) was exhibited for shootlet explants cultured on MS medium supplemented with 1.5 mg/l NAA (Figure 4A). The least number of roots per explant (nil) was shown in hormone-free medium (Table 2). Furthermore, numerous very fine and hairy like false roots were produced on MS medium supplemented with NAA auxin. The results affirmed that using

high concentration of NAA allowed shootlet explants to form roots at greatest number; thus these conditions would be recommended for optimal root formation. As general observation, the addition of NAA into the media at any of the tested concentrations resulted in a significant increase in number of roots regenerated from the explants as compared to the IBA. These findings are similar with reports of Khalafalla *et al*. (2007) who found 12.3 ± 1.7 roots per shootlet explant at the highest concentration of NAA (1.0 mg/l) tested after two weeks of culturing.

Table 2. Effects of various concentrations of IBA and NAA for the formation of roots on the *in vitro* grown microshoots of cactus pear after two weeks of culturing.

Auxins (mg/l)		Rooting response (%)	Main root number per explant (n)	Root length per explant (cm)	Root fresh weight per explant (g)	Root dry weight per explant (g)
IBA	NAA					
0.0	0.0	0.00	0.000	0.000	0.000	0.000
0.25	0.0	100	3.75g	6.23c	1.070c	0.070cd
0.5	0.0	100	4.75e	7.08b	1.525b	0.098b
1.0	0.0	100	3.00h	10.50a	1.488b	0.075c
1.5	0.0	100	4.25f	10.75a	1.880a	0.140a
0.0	0.25	100	3.75g	2.00e	0.753e	0.013f
0.0	0.5	100	6.25c	1.33gh	1.020c	0.065d
0.0	1.0	100	5.25d	1.60fg	0.670e	0.013f
0.0	1.5	100	10.00a	1.13h	1.157c	0.073cd
0.25	1.5	100	9.65b	1.80ef	1.000cd	0.050e
1.5	0.25	100	3.98fg	5.70d	0.825de	0.045e
CV (%)		0.00	4.60	5.31	8.01	11.94

Treatment means within a column followed by the same letter (s) are not significantly different according to Duncan's Multiple Range Test (α = 0.01).

All the tested auxin types and concentrations in Table 2 in the present study allowed the root to grow up in the length ranged between 0.0 to 10.75 cm with highly significant differences ($p<0.01$) among these values. Of the two auxins tested, IBA at 1.0 and 1.5 mg/l induced the highest mean root length (10.75 and 10.5 cm) per shootlet explant after two weeks of culture, respectively (Figure 4B). There was no statistical difference between these two concentrations regarding root length. In this regard, root length was generally observed to be increased with the simultaneous increase in the concentration of IBA from 0.25 to 1.5 mg/l. The maximum root length achieved on the NAA auxin was 2.0 cm at 0.25 mg/l (Table 2). As general observation, media supplemented with IBA significantly increased the root length as compared to NAA.

The maximum mean root fresh weight (1.88 g) and root dry weight (0.14 g) per explant was obtained on medium containing 1.5 mg/l IBA (Table 2). The highest root fresh weight and root dry weight value per explant obtained from the medium supplemented with NAA were 1.157 g and 0.073 g,

respectively which are smaller as compared with that obtained on IBA. In contrary, the lowest root fresh weight and root dry weight per explant was recorded on medias fortified with 1.0 mg/l NAA followed by 0.25 mg/l NAA. Statistically non-significant mean root fresh weights were recorded between mediums fortified with 0.5 mg/l and 1.0 mg/l IBA (Table 2).

Conclusion

Maximum shoot proliferation was obtained when explants were cultured on MS medium supplemented with a relatively low level of Kinetin concentrations (at 1.0 mg/l) for most of the growth parameters tested. In the case of root induction stage, the highest mean main root number per explant was achieved when the *in vitro* derived shoots were cultured on MS medium supplemented with NAA at 1.5mg/l.

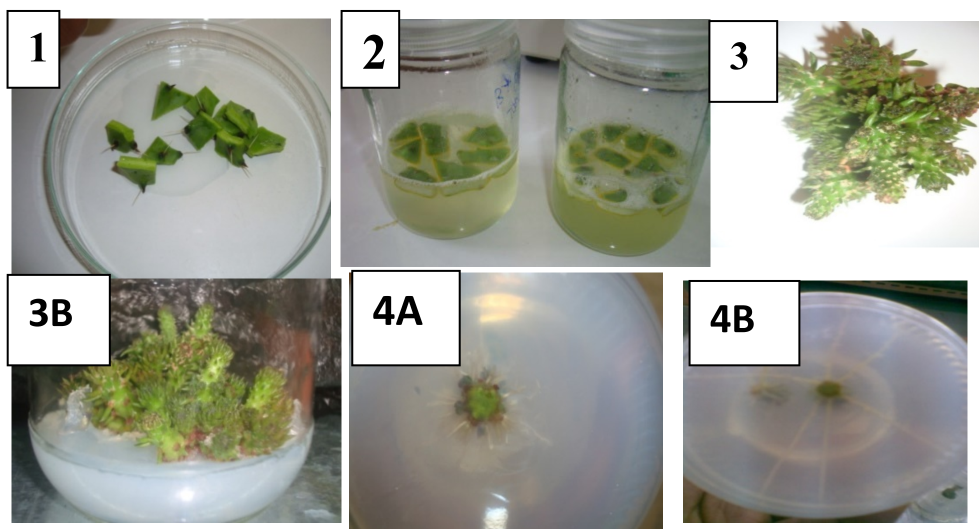

Figure 1-4. *In vitro* micropropagation system for cactus pear (*Opuntia ficus-indica*). (1) Preparation of explants with areoles; (2) Sterilization of explants in commercial clorox (NaOCl); (3A) *In vitro* regerated shoots on MS medium supplemented with BA (2.0 mgl^{-1}) after 12 weeks of culturing; (3B) Multiple shoot proliferation on MS medium supplemented with Kin (1.0 mgl^{-1}) after 12 weeks of culturing; (4A) *In vitro* rooted plantlets on MS medium supplemented with NAA (1.5 mgl^{-1}) after two weeks of culturing; (4B) *In vitro* rooted plantlets on MS medium supplemented with IBA (1.5 mgl^{-1}) after two weeks of culturing.

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