IN VITRO REGENERATION OF CACTUS (*Opuntia monacantha* L.)

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CERTIFICATE

This is to certify that the thesis entitled **"*IN VITRO* REGENERATION OF CACTUS (*Opuntia monacantha*)"** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* **MASTER OF SCIENCE** in **BIOTECHNOLOGY** *embodies the results of a piece of bona fide research work carried out by* **ASHIQUZZAMAN KHAN** *bearing Registration. No.* **13-05450** *under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information as has been availed of during the course of this investigation has duly been acknowledged.

Dated:
Dhaka, Bangladesh

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Dedicated to
my beloved parents
and
my sister

ABBREVIATIONS AND ACRONYMS

Acronym		Full form
Agril.	:	Agriculture
Biol.	:	Biological
cm	:	Centimeter
CRD	:	Completely Randomized Design
DMRT	:	Duncan's Multiple Range Test
Conc.	:	Concentration
DAI	:	Days After Inoculation
et al.	:	And others (at elli)
FAO	:	Food and Agricultural Organization
IASC	:	International Aloe Science Council
g/L	:	Gram per litre
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	*a*- Napthalene acetic acid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
Int.	:	International
2-iP	:	2-isopentenyladenine
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
PVP	:	Polyvinylpyrrolidone
PLB	:	Protocorm-like bodies
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Et cetera
WAI	:	Weeks After Inoculation
PSI	:	Pound per square inch

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June, 2020

SAU, Dhaka **The Author**

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IN VITRO REGENERATION OF CACTUS (*Opuntia monacantha*)

ABSTRACT

The present study was carried out at the laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period from January, 2019 to December, 2019 to determine the evaluation of the effect of different plant growth regulators (BA and IBA) on *in vitro* plantlet regeneration of Cactus. The young cladodes of Cactus were used as explants and they were cultured on Murashige and Skoog medium (MS media) supplemented with Benzyladenine (BA) with different concentrations (1.0, 1.5, 2.0, 2.5 and 3 mg/L) and Indole-3-butyric acid (IBA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) either alone or in combination of both. The experiments were arranged in Completely Randomized Design (CRD) with five replications. The highest shoot number (4.4, 5.4 and 6.6 cm) and shoot length (2.58 , 5.1 and 7.18 cm) was found in 2.5 mg/L BA at 2 WAI, 3 WAI and 4 WAI, respectively. The maximum number of roots (4.8, 6.4 and 6.8) were recorded from 2.0 mg/L IBA supplemented media. The maximum shoot induction (94.6%) in minimum days (5.2) was observed with 2.5 mg/L BA+2.0 mg/L IBA. The highest number of shoots (4.2, 5.2 and 7.0) were found in 2.5 mg/L BA+2.0 mg/L IBA. The highest number of roots induction (94.6%) was recorded with 2.5 mg/L BA + 2.0 mg/L IBA supplemented media in minimum days (5.2). The highest length of root (3.4, 5.7 and 7.30 cm) at 2 WAI , 3 WAI and 4 WAI, respectively was found in 2.5 mg/L BA + 2.0 mg/L IBA. The survival rate of regenerated plantlets were 100% in growth chamber, 90% in shade house and 100% in field condition. Therefore, an effective protocol had been developed for *in vitro* regeneration of Cactus which can meet up the great commercial demand for the aesthetic, medicinal and fruit need for the year round production in Bangladesh and can act as the strategic tool to combat desertification in arid and semi arid regions.

CHAPTER - I

INTRODUCTION

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INTRODUCTION

The Cactus (*Opuntia monacantha*), commonly known as Drooping prickly pear, Cochineal prickly pear, or Barbary fig, is a species of plant in the family *Cactaceae*. The species was first formally described in 1812 by botanist Adrian Haworth in Synopsis *Plantarum Succulentarum* (Taylor *et al*., 2013). It is native to the American continent and comprises more than 2,000 species that are primarily distributed over four diversity centers in arid and semiarid regions (Hernandez *et al*., 2001). In Mexico, in particularly, 68% species of *Cactaceae* may be found. Columbus brought these plants to Europe, and later Linneus named them as Cacti (Serrano and Silva, 2008). The *Cactaceae* family includes approximately 130 genera. Of these, the *Opuntia* and *Nopalea* genera are the most important due to their usefulness to man (Valdez and Osorio, 1997). Cactus plants have a distinct anatomy and physiology as gross shape; buds that turn into areoles, which are the main character of Cacti. These plants open their stomatas and take up CO_2 at night, when temperatures are lower and humidity higher than during the day time. This invariably results in reduced water loss which offers exceptional possibilities for large quantities of biomass in water limited areas that are useful for livestock feed (Ogburn and Edwards, 2010).

Besides, Cacti have evolved to resist arid weather, and since these plants can preserve and maintain water in their parenchyma, since they contain fewer respiratory structures, produce serum secretions, and have plunged stomas; they are thus considered as xerophytic plants. As mentioned above, areoles are cacti-specific structures found on the ribs or tubers as a group of wooly hairs or spines, which can develop buds, flowers or branches (Glass, 1998). Charles Darwin was the first to note that these cacti have thigmotactic anthers; when the anthers are touched, they curl over, depositing their pollen. This movement can be seen by gently poking the anthers of an open *Opuntia* flower. The same trait has evolved convergently in other species (e.g. *Lophophora*).

Prickly pear (*Opuntia monacantha*) also produce an edible fruit, commonly eaten in Mexico and in the Mediterranean region, commonly called Cactus fruit, Cactus fig, Indian fig, Nopales or Tuna (Grigson, 2007). The fruit can be red, wine-red, green, or yellow-orange. Raw *Opuntia* leaves contain 88% water, 10% carbohydrates, and less than 1% both of protein and fat. In a 100 gm reference amount, raw leaves provide 41 calories, 17% of the Daily Value (DV) for vitamin C, and 24% DV for magnesium. Prickly pears are often used to make appetizers, soups, salads, entrees, vegetable dishes, breads, desserts, beverages, candy, jelly, and drinks (Midey, 2005). The young stem segments, usually called nopales, are also edible in the most species of *Opuntia*. They are commonly used in Mexican cuisine in dishes such as eggs with Nopales, or tacos with Nopales. Nopales are also an important ingredient in New Mexican cuisine. It was introduced as a cheaper alternative to corn for the production of tortillas and other corn products (Hunter, 1985).

Opuntia contains a range of phytochemicals in variable quantities, such as polyphenols, dietary minerals and betalains. Identified compounds under basic research include gallic acid, vanillic acid and catechins, (Guzman-Maldonado *et al*., 2010). The Sicilian prickly pear contains betalain, betanin, and indicaxanthin, with highest levels in their fruits (Butera *et al*., 2002).

Long historical and worldwide use of medicinal plants and phytochemicals has demonstrated the efficacy of traditional medicine to prevent the onset and progression of chronic diseases. Among the number of plants identified and used in folk medicine, *Opuntia* species exhibit a lot of beneficial properties and high biotechnological potential. They have been used for centuries as food resources and in folk medicine for the treatment of chronic diseases and many other illnesses. Some species are cultivated for economical and medicinal purposes in Mexican area.

Though differences in the phytochemical composition exist between domesticated and wild *Opuntia* spp., the presence of antioxidants (flavonoids, ascorbate), pigments (carotenoids, betalains such as indicaxanthin), or phenolic acids has been reported in all *Opuntia* products, including seeds, roots, pears, cladodes or juice. Antioxidants could be responsible for the nutritional and protective benefit of *Opuntia* enriched diets in chronic diseases, in which inflammation and oxidative stress play a major involvement. Other plant materials such as biopeptides or soluble fibers have been characterized and contribute to the medicinal properties of *Opuntia* spp. It was an update on the active compounds and the biological and medical benefit of wild and domesticated *Opuntia* spp. in chronic diseases. Its pulp and juice are considered as the treatments for wounds and inflammation of the digestive and urinary tracts (Maran *et al*., 2013).

In one recent study, it was found that *Opuntia* aided in the prevention or slow down of diabetes, obesity, metabolic syndrome, cardiovascular disease, and some cancers. The results of the group that was taking *Opuntia* showed a reduction in BMI, body composition, and waist circumference when compared to the placebo group (Mayes *et al*., 2012).

Besides these, Cactus is used as a fodder crop for animals in arid and dryland regions. The thick skin of Nopal cactus can be harvested as an environmental friendly leather replacement which is commonly known as vegan leather. Bioethanol can be produced from some *Opuntia* species (Ciriminna, 2017). Nopal juice can be used to produce bioplastic.

There are three primary markets for cacti: nurseries, supermarkets and private collectors. Nurseries supply cacti for residential gardens, commercial establishments (e.g., golf courses) or public areas, such as parks. Xeriscaping or landscaping with plants adapted to arid climates is gaining popularity in desert cities where water shortages are a growing concern. Due to their large showy appearance, the species most frequently used in landscaping projects are

Barrel cactus (*Ferocactus* spp.), Prickly pear cactus (*Opuntia* spp.) and Saguaro cactus. In supermarkets, large quantities and varieties of miniature Cacti are grown from seeds. Private collectors represent a specialized market that is primarily interested in rare or newly discovered species within the *Cactaceae* family with high commercial value, such as *Pelecyphora strobiliformis*, *Strombocactus disciformis*, *Geohintonia Mexicana* and *Mammillaria luethyi* etc.

Like many other countries, in Bangladesh different government and non-government organizations are working in this area. Many agricultural, medicinal, forest crops are cultivated and multiplication was done by tissue culture technique. Cactus is one of them. Many plant breeders of different countries as well as Bangladesh have been employing biotechnological tools for the improvement of Cactus. In Bangladesh, Cactus are grown scatteredly all over the country, but there is no statistics available in our country about area and production of Cactus. Presently, some companies have started cultivating and marketing Cactus. More than 46 species of cacti (for instances, Golden barrel, Old man, *mammillaria*, *acanthocalycium*, *lobivia*, *gymnocalycium*, Victoria, *melocactus*, *opuntia*, bird nest, *ferocactus*, *cleistocactus* and much more) are now found in different nurseries.

With this precarious perspective, propagation should be a tool to maintain these valuable genetic resources. However, seed propagation is insufficient to satisfy the demand for cactus due to limited production, availability and viability of this reproductive material. Moreover, vegetative propagation is insufficient. Consequently, *in vitro* techniques are fundamental tools to satisfy the demand. More importantly, since numerous Cacti species are threatened or endangered, *in vitro* micropropagation has become, since Mauseth's work in 1976, the primary tool for preserving these valuable genetic resources. Cacti can be propagated via rooting of cuttings, sprouts and grafts. However, some wild species with monopodial development present serious difficulties by vegetative propagation, mainly due to few or a lack of production of sprouts which grow

slowly and, in some cases, the small size of seeds, and low viability (Soltero, 1996). As an important ornamental plant, Cactus may be a source of income for domestic uses as well as for export. Therefore, attempt was made to develop protocols for large scale *in vitro* propagation of locally available Cactus (*Opuntia monacantha*). Considering the above facts, the present investigation was undertaken to find out the performance of different hormones with the optimum concentration of BA and IBA for *in vitro* plantlets regeneration of Cactus.

The specific objectives were:

1. To establish the *in vitro* regeneration protocols of Cactus,
2. To standardize the hormonal effect for the *in vitro* response of Cactus,
3. To select the best hormonal concentration for *in vitro* regeneration of Cactus, and
4. To study the rapid multiplication of Cactus within a short time period.

CHAPTER - II

REVIEW OF LITERATURE

CHAPTER II
REVIEW OF LITERATURE

In vitro micropropagation of *Opuntia monacantha* was investigated through the present study. The regeneration of Plantlets from the culture media of cladodes explants followed by genetic stability of the plantlets seems to be meager. However, available information in this regards were reviewed and presented in this sections.

2.1 *In vitro* regeneration of Cactus

Plant tissue culture is the newest route in the science of cell biology. Tissue culture is the process of regeneration in an artificial nutrient medium under aseptic condition. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. Development of new organized structures i.e. organs from the old one through tissue culture is done by two ways: direct and indirect. Emergence of adventitious organs directly from explants known as direct method. Indirect is the process of regeneration new organs through shoot and root formation (Ramirez-Carvajal *et al*., 2009).

In cases where overall collection in natural habitats has occurred, the starting material for cactus propagation is very limited or scarce. Micropropagation can be a useful tool for solving or overcoming these problems because sexual propagation is replaced by vegetative propagation cycles, which usually maintain the genetic fidelity of the starting material (cloning) and shorten the propagation period (usually from several months to a few weeks) (Malda *et al*., 1999). In general, artificial growth media provide good conditions for either *in vitro* seed germination of almost all Cactus species or for their micropropagation under aseptic conditions and controlled temperature, light and nutrition regimes (Shedbalkar *et al*., 2010). In some cases, full-strength MS (Murashige and Skoog, 1962) medium can inhibit or delay seed germination of Cactus species (Lema-Ruminska and Kulus, 2014).

Micropropagation of any plant species involves the selection of the explant donor followed by the elimination of surface microbial contamination of the tissues, inoculation in an appropriate culture media and incubation under controlled conditions to produce vegetatively a large number of plants. One of the primary issues in establishing *in vitro* cultures from outdoors or in greenhouse maintained Cactus plants is the elimination of bacterial and fungal contamination. Cactus plants grown outdoors or under greenhouse conditions may be used as explant sources to establish *in vitro* cultures; alternatively, *in vitro* plants raised from seeds had also been used as starting material for micropropagation of Cacti (Lema-Ruminska and Kulus, 2012).

2.2 Sterilization of explants

Both NaOCl and $HgCl_2$ are oxidizing agents and damage the microorganism by oxidizing the enzymes. The ineffectiveness of NaOCl may be due to the reason that it is a mild sterilizing agent (Sirivastava *et al*., 2010). $HgCl_2$ is reported a better sterilizing agent as compared to NaOCl but is more toxic and requires special handling and is difficult to dispose (Maina *et al*., 2010).

Clayton *et al*. (1990) established *in vitro* cultures from shoot-tip explants (1-2 cm in diameter) from primary or secondary stems of the rare or endangered species *Mammillaria wrightii*. Explants were disinfected by immersion in 95 % ethanol for 1 min then in a 2 % commercial sodium hypochlorite (NaClO) solution for 7 min and were finally subjected to rinsing three times in sterile water.

Sriskandarajah and Serek (2004) also started *in vitro* cultures using phylloclade explants of *Schlumberguera* and *Rhipsalidopsis* from plants grown in the greenhouse. Due to the contamination issue frequently faced during the establishment of *in vitro* cultures of cacti species, many investigators had started from *in vitro* aseptic seedlings or other secondary explants excised from *in vitro* cultures.

Khalafalla *et al*. (2007) found sterilization of young Cactus cladodes sprayed with 70% ethanol and then immersed in 15% sodium hypochlorite for 20 min, and then rinsed in sterile distilled water for three times give best result.

Estrada-Luna *et al*. (2008) used axillary buds from young cladodes (4-5 cm in length) excised from ornamental prickly pear *Opuntia lanigera* Salm-Dyck plants grown in a greenhouse to establish a micropropagation protocol. Spine-hairs were eliminated from the cladodes using scissors and they were treated with 70 % ethanol for 5 min and then immersed for 30 min in a commercial bleach solution (6 % NaClO) containing 0.1 % Tween-20. The cladodes were then washed with deionized sterile water (five times). Pairs of areoles were dissected from the cladodes and were used as explants.

Jagatheeswari and Ranganathan (2012) found sterilization of explants with lower concentration of mercuric chloride treatments with lesser timings gave the best result.

2.3 Multiple shoots induction

Clayton *et al*. (1990) reported axillary shoot regeneration from shoot-tip explants of the rare or endangered the cactus species *Escobaria missouriensis* (Sweet), *Sclerocactus spinosior*, *Toumeya papyracantha*, *Mammillaria wrightii*, *Pediocactus bradyi* and *Sclerocactus mesaeverdae*, cultured on MS, B5, SH and L2 basal media (Gamborg *et al*., 1968, Schenk and Hildebrandt, 1972 and Phillips and Collins, 1979) or MS basal medium supplemented with different cytokinins (adenine sulfate, BA, 2iP, Kinetin and zeatin) and auxins (IAA, IBA, NAA and 4-amino-3,5,6- trichloropicolinic acid). They observed that the basal L2 medium was superior, but still MS with low or moderate to high cytokinin concentrations (22.8 to 49.2 µM) is the most popular and induced the greatest shoot formation. For all species tested, the combination 45.6 µM zeatin + 1.1 µM NAA was optimal for axillary shoot production.

Machado and Prioli (1996) reported the production of axillary shoots from *Cereus peruvianus* areoles starting from lateral explants cultured on MS

medium containing 4.44 µM BA and 5.71 µM IAA or 5.37 µM NAA.

Perez-Molphe-Balch *et al*. (1998) reported that *in vitro* cultures of *Astrophytum myriostigma*, *Cephalocereus senilis*, *Coryphantha clavata*, *C. durangensis*, *Echinocactus platyacanthus*, *Echinocereus dubius* (Engelm.), *E. pectinatus*, *Echinofossulocactus* spp., *Ferocactus hamatacanthus*, *F. latispinus*, *F. pilosus*, *Mammillaria candida*, *M. craigii*, *M. Formosa*, *M. obscura*, *M. sphacelata*, *M. uncinata*, *Nyctocereus serpentinus*, *Stenocactus coptonogonus* and *A. Berger* exhibited multiple shoot formations from areoles on MS culture medium with 4.4 or 8.9 µM BA alone or in combination with 0.54 or 5.4 µM NAA.

Wakhlu and Bhau (2000) induced callus from the pith tissue of *Coryphantha elephantidens*, inoculated onto MS growth medium with 9.1 µM 2,4-D and 2.3 µM Kinetin, and subsequently regenerated shoots after four weeks of incubation on growth medium supplemented with 6.9 µM kinetin and 2.3 µM 2,4-D. The callus tissue retained its regenerative capability over a lengthy period (18 subcultures), and the regenerated plants were morphologically similar to the explant donor plants.

Papafotiou *et al*. (2001) reported indirect shoot regeneration from calli formed in tubercle explants of *Mammillaria elongate*, cultured on MS medium with 1.1 µM NAA and 22.2 µM BA.

Perez-Molphe-Balch and Davila Figueroa (2002) established *in vitro* cultures of the Mexican endangered Cacti *Pelecyphora aselliformis* and *Pelecyphora strobiliformis*, and they observed shoot formations from hormone activated areoles after 60 days of incubation on MS medium in the presence of different cytokinins. BA was more effective than either 2iP or thidiazuron for activating axillary buds. *P. aselliformis* and *P. strobiliformis* produced 13.7 and 12.4 shoots per apical explant, respectively, in the presence of 8.8 µM BA.

Giusti *et al*. (2002) investigated the responses of explants from *Escobaria minima, Mammillaria pectinifera* and *Pelecyphora aselliformis*, cultured on MS medium containing 2.3 µM thidiazuron; they observed callus formation and then shoot regeneration (3.0, 3.5 and 10.2 shoots per explant of each species, respectively). Interestingly, Poljuha *et al*. (2003) reported that *in vitro* propagated shoots of *Mammillaria gracillis*, developed callus spontaneously, which in turn regenerated normal and hyperhydric shoots in the absence of exogenous growth regulators.

More recently, Garcia-Rubio and Malda-Barrera (2010) described a micropropagation protocol for the endangered endemic cactus *Mammillaria mathildae*, using basal explants that produced callus on MS medium containing combinations of BA (0.0, 22.2 and 44.4 µM) and IAA (0.0, 1.4, 2.9 and 5.7 µM), from which shoots were regenerated.

Davila-Figueroa *et al*. (2005) achieved multiple shoot formations in eight species of *Turbinicarpus* cultured on MS medium containing cytokinins with efficiencies ranging from 7.8 shoots per explant for *T. valdezianus*, 19.7 for *T. pseudopectinatus*, using BA or 2iP.

Retes-Pruneda *et al*. (2007) developed micropropagation systems by areole activation for *Echinocereus knippelianus*, *E. schmollii*, *Mammillaria carmenae*, *M. herrerae*, M. theresae Cutak, Melocactus curvispinus, Escontria chiotilla and *Polaskia chichipe*, using MS medium containing BA (0.0, 2.22, 4.44 and 8.88 µM) or 2iP (0.0, 4.92, 14.8 and 24.6 and 24.6 µM). The greatest responses were obtained with the highest cytokinin levels.

Estrada Luna *et al*. (2008) developed a micropropagation protocol for the ornamental Prickly pear cactus *Opuntia lanigera*. The explants tested were placed in vertical or horizontal positions on MS medium with different cytokinins (0.0, 5.55, 11.1, 22.2 and 31.1 µM BA, 0.0, 6.15, 12.3, 24.6 and 34.4 µM 2iP and 0.0, 5.7, 11.4, 22.8 and 31.9 µM Kin) for the activation of areoles to produce axillary shoots. It was observed that vertical orientation was

optimal (approximately 5 shoots per explant) compared with horizontal (3.7 shoots per explant) and that the highest shoot regeneration was achieved with BA (8 shoots per explant) compared with 2iP and Kin (2 shoots per explants)

Cardarelli *et al*. (2010) observed multiple shoot formations from areoles of the endangered cactus *Obregonia denegrii*, on MS medium containing 4.4 µM BA and 10.7 µM NAA together with N-(2-chloro-4-pyridyl)-Nphenylurea (4-CPPU), a type of cytokinin.

Ruvalcaba-Ruiz *et al*. (2010) tested half-strength MS medium supplemented with BA (0.0, 4.44, 8.88 and 13.3 µM) and NAA (0.0, 2.69 and 5.37 µM) to micropropagate the Mexican endemic and endangered ornamental cactus *Coryphanta retusa*. The highest shoot regeneration was recorded in the presence of 8.88 µM BA after 60 days of incubation.

Rosa-Carrillo *et al*. (2012) reported micropropagation following activation of areoles in tissue cultures of 14 species and subspecies of *Turbinicarpus*, with efficiencies of 4.0 shoots per explant for *T. hoferi* in the presence of 4.4 µM BA and of 26.5 µM BA for *T. pseudomacrochele*, with 3.3 µM BA after 60 ± 5 days of incubation.

2.4 Elongation of shoots

Rajam (2006) reported that the efficient shoot induction has been observed but elongation of shoots into proper shoots is a consistent problem. With a low concentration of BA or kinetin to assess its effect on shoot proliferation. They suggested that BAP might increase shoot proliferation by inhibiting the transport of auxins leading to a more favorable balance between cytokinins and auxins but they did not get good result with respect to elongation.

2.5 Multiple roots induction

Kelly (2009) reported that vegetative propagation relies on the plant's ability to produce new roots from an existing part of the plant such as a stem or leaf. Kuti (1998) reported that root dynamics of *Opuntia* species have been shown to be

related to their water potentials under water limiting conditions.

According to Synman (2006) placing the cladodes flat on the media, more areoles came in contact with the media and therefore more roots developed in an average of only 3.4% areole complexes not rooting. Side roots grew as much as 8 and 5 mm per day for *O. ficus* and *O. robusta*, respectively. Rain roots, grew up to 7 and 5 mm within a day for *O. ficus* and *O. robusta*, respectively.

Akram *et al*. (2013) reported that rooting initiation started within 7 days and 100% areole rooting was obtained on MS basal medium, under a 16 hr photoperiod on the 17^{th} day of culture. Result demonstrated that there was a significant difference between 17^{th} and 21^{th} days of culture. The highest root length (56 mm) and root number (5.2) were achieved after 17 days. Several studies revealed that rooting is favored in most plant species when auxins are available in the culture media (Hartmann *et al*., 1997), however, several *Opuntia* species have successfully been rooted and normally grown without auxins (Escobar-Araya *et al*., 1986, Estrada-Luna, 1988, Mohamed-Yasseen *et al.*, 1995, Garcia- Saucedo *et al*., 2005 and Estrada *et al*., 2008).

Khalafalla *et al*., (2007) obtained highest percentage of root using IAA at 0.5 mg/L and satisfactory rooting occurred for three *Opuntia* genotypes treated with 2 mg/L IBA.

Mauseth (1979) reported that plant growth regulators (PGRs) had also been shown to influence organ differentiation in *Opuntia*. Exogenous Gibberellin (GA3) inhibit the development of daughter cladodes (Nobel, 1995). On the contrary, Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) were shown to have a positive effect on root development (Lazcano *et al*.,1999 and Mulas *et al*., 1992). Under *in vitro* condition, IBA was reported to produce more roots in *O. ficus* and other species of *Opuntia* (Mohammed-Yasseem *et al*., 1995 and Juarez-Passera, 2002).

Perez-Molphe-Balch *et al*. (1998) and Resende *et al*., (2010) reported that when the produced shoots were greater than 5 mm in length and diameter, which usually took about 8–12 weeks after inoculation on the proliferation media, they were transferred, for 3–5 weeks, onto the rooting medium containing 2.5 –6.0 μM IAA or IBA and possibly half-strength MS (Rosas *et al*., 2001 and El Finti *et al*., 2013). Sometimes, due to a high level of endogenous auxins, rooting can occur spontaneously without PGRs (e.g. *Opuntia* spp., *Cephalocereus senilis*, *Coryphantha elephantidens*), eliminating the need for rooting medium and reducing costs and time (Clayton *et al*., 1990 and El Finti *et al*., 2012).

El Finti *et al*. (2012) reported that the rooting of *in vitro* generated shoots was achieved most efficiently on half-strength MS basal medium supplemented with 0.5 mg/L of IBA or IAA and with rooting frequencies were in the range from 95 to 100% and the highest mean number of root (19.1) was obtained with IBA. Currently IBA is the most widely used auxin to stimulate rooting in many plant species because of its high ability to promote root initiation.

2.6 Acclimatization

Due to disruptions in photosystem II in plants grown in tissue culture under reduced (in comparison to natural) light conditions (Balen *et al*. 2012), such plants cannot be transferred directly into outdoor conditions. First microcuttings must be acclimatized for about 4 weeks to *in vivo* conditions in a light sand substrate (peat:perlite 1:2, sand:soil 1:1, or peat:sand 1:1 by volume) in the growth chamber (Papafotiou *et al*., 2001 and Resende *et al*., 2010) to harden them to higher temperatures and light intensity and lower air moisture in comparison with the culture vessel environment. Afterwards, plantlets can be transferred to *in vivo* conditions (Quiala *et al*., 2009). This, however, may be difficult, for Cacti like other plants are greatly influenced by the controlled conditions in tissue culture, which may result in the death of plantlets (Balen *et al.,* 2012).

Akram *et al*. (2013) reported that the acclimatization procedure applied to *in vitro* regenerated plantlets was successful, plantlets showing a 100% survival when transferred to pots containing pit and perlite (2:1). After transplantation, plantlets survival were 100%, which was similar to the reports for other Prickly pear cactus species (Escobar-Araya *et al*., 1986, Mohamed-Yasseen *et al*., 1995, Juarez and Passera, 2002 and Estrada *et al*., 2008) and most micropropagated Cacti (Ault and Blackmon, 1987, Clayton *et al*., 1990 and Johnson and Emino, 1979).

CHAPTER - III

MATERIALS AND METHODS

CHAPTER III
MATERIALS AND METHOD

3.1 Time and location of the experiment

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of January, 2019 to December, 2019.

3.2 Experimental materials

3.2.1 Source of materials

The planting materials of *Opuntia monacantha* (Cactus) were collected from Banglar Nursery, West Manikdi, Dhaka Cantonment, Dhaka-1206.

3.2.2 Plant materials

Healthy young cladodes of cactus about 1-3 cm in length were collected and excised from donor plants which were previously cultured under greenhouse conditions.

Plate 1. Donor Cactus plant for explants collection

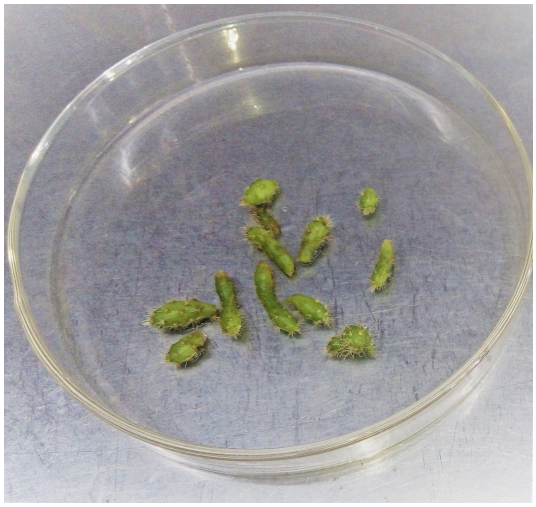


Plate 2. Cladodes of Cactus

Fresh, healthy and disease free young cladodes of cactus were harvested in a beaker filled with water. The explant were washed thoroughly with running tap water for removing soil. Young cladodes were trimmed to size of 1-3 cm for further use as explants.

3.2.3 Instruments

Metal instruments viz., forceps, scalpels, needles, spatulas, aluminum foils, tissue, cotton, plastic caps etc. were used as instruments and Erlenmeyer flasks, culture vials, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 mL, 50 mL, 100 mL, 500 mL and 1000 mL) etc. were used as glassware. All instruments were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glassware

In all the experiments the borosil glassware was given priority. The glassware were first rinsed with the liquid cleaners and then washed thoroughly with tap water before the detergent (Trix) was removed completely. Then set up to autoclave for sterilization.

3.2.5. Culture media

The degree of success in tissue culture is mainly related to the choice of nutritional components and growth regulators. Presence of plant growth

regulators plays a significant role in a successful regeneration of any plant species. Media for tissue culture should contain all major and minor elements, vitamins and growth regulators which are essential for normal plant growth. Explants were inoculated onto media composed of basal MS medium supplemented with the plant growth regulators. Composition of MS media has been shown in (Appendix I). Hormones were added separately to different media according to the requirements.

3.2.6 Sterilization of Instruments and Glassware

All the glassware and instruments were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Then the glassware and instruments were sterilized in an autoclave at a temperature of 121°C and at 1.06 kg/cm^2 (15 PSI) pressure for 30 minutes.

3.3 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins, growth hormones etc. were used.

3.3.1 Stock solution A - Macronutrients

Stock solution of macronutrients was prepared up to 10 times the concentration of the final medium in 1000 mL of distilled water (DW). Ten times the weight of the salts required per litre of the medium were weighed properly and dissolved by using a magnetic stirrer in about 750 mL of distilled water and then made up to 1000 ml by further addition of distilled water (DW). To make the solution free from all sorts of solid contaminants, it was filtered through Whatman no. I filter paper. Then it was poured into a plastic container, labeled with marker and stored in a refrigerator at 4 °C for later use.

3.3.2 Stock solution B - Micronutrients

The stock solution of micronutrients was made up to 100 times the final strength of necessary constituents of the medium in 1000 mL of distilled water (DW) as described for the stock solution of macronutrients. The stock solution was filtered, labeled and stored in a refrigerator at 4 °C.

3.3.3 Stock solution C - Iron sources

This was prepared at 100 times the final strength of Fe_2SO_4 and Na_2 EDTA in 100 mL of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 mL by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4 °C.

3.3.4 Stock solution D - Vitamins

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 mL of distilled water. Then the final volume was made up to 1000 mL by further addition of distilled water. The solution was dispensed into 10 mL aliquots and stored at 20°C. Myo-inositol was used directly at the time of media preparation.

3.3.5 Stock solution E - Hormone

The first step of the preparation of the medium was the preparation of hormone stock solutions. To expedite the preparation of the medium separate stock solutions for growth regulators were prepared and used. Growth regulators and concentrations used for *in vitro* regeneration of are presented below:

1. BA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) for shoot induction
2. BA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) combined with IBA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) for shoot and root formation respectively
3. IBA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) for root induction

3.4 Preparation of the hormonal stock solution

To prepare the above hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Generally, cytokinins were dissolved in few drops of basic solutions (1N NaOH) and auxins were dissolved in few drops of basic solutions (1N NaOH) or 70% ethyl alcohol.

Hormone (solute)	Solvents used
BA	1N NaOH
IBA	96% ethyl alcohol

In present experiment, the stock solution of hormones were prepared by following procedure.

3.4.1 Stock solution of BA

A 100 mg of powder hormone was placed in a small beaker and then dissolved in 10 mL of 1N NaOH solvent. Finally the volume was made up to 100 mL by the addition of sterile distilled water using a measuring cylinder.

3.4.2 Stock solution of IBA

A 100 mg of powder hormone was placed in a small beaker and then dissolved in 10 mL of 96% ethyl alcohol solvent. Finally the volume was made up to 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4°C for use up to two month.

3.5 Treatments

Four sub experiments were conducted to assess the effect of different concentrations of BA and IBA on shoot proliferation and subsequent rooting of the multiplied shoot.

3.5.1 Sub-experiment - 1

Effect of BA on *in vitro* shoot induction potentiality in Cactus

Five levels of BA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) and control (0.0 mg/L)

treatments were used. The experiments were arranged in Completely Randomized Block (CRD) with three replications.

3.5.3 Sub-experiment - 2

Effect of IBA on root induction potentiality of *in vitro* regeneration in Cactus

Five levels of IBA (0.5, 1.0, 1.5, 2.0 and 2.5mg/L) and control (0.0 mg/L) were used. The experiments also practiced as sub-experiment- 1.

3.5.2 Sub-experiment - 3

Combined effect of BA and IBA on *in vitro* shoot and root induction potentiality in Cactus

In this sub-experiment, Five levels of IBA (0.5, 1.0, 1.5, 2.5 and 3.0 mg/L) were practiced with each level of BA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). Total 25 combinations of BA and IBA were examined in this experiment and control treatment also practiced. The combine treatments were as follows:

T1 = BA 1.0 mg/L + 0.5mg/L IBA

T2 = BA 1.0 mg/L + 1.0 mg/L IBA

T3 = BA 1.0 mg/L + 1.5 mg/L IBA

T4 = BA 1.0 mg/L + 2.0 mg/L IBA

T5 = BA 1.0 mg/L + 2.5 mg/L IBA

T6 = BA 1.5 mg/L + 0.5 mg/L IBA

T7 = BA 1.5 mg/L + 1.0 mg/L IBA

T8 = BA 1.5 mg/L + 1.5 mg/L IBA

T9= BA 1.5 mg/L + 2.0 mg/L IBA

T10= BA 1.5 mg/L + 2.5 mg/L IBA

T11= BA 2.0 mg/L + 0.5mg/L IBA

T12 = BA 2.0 mg/L + 1.0 mg/L IBA

T13 = BA 2.0 mg/L + 1.5 mg/L IBA

T14 = BA 2.0 mg/L + 2.0 mg/L IBA

T15 = BA 2.0 mg/L + 2.5 mg/L IBA

T16 = BA 2.5 mg/L + 0.5mg/L IBA

T17 = BA 2.5 mg/L + 1.0 mg/L IBA

T18 = BA 2.5 mg/L + 1.5 mg/L IBA

T19= BA 2.5mg/L + 2.0 mg/L IBA

T20= BA 2.5 mg/L + 2.5 mg/L IBA

T21= BA 3.0 mg/L + 0.5 mg/L IBA

T22= BA 3.0 mg/L + 1.0 mg/L IBA

T23= BA 3.0 mg/L + 1.5 mg/L IBA

T24= BA 3.0 mg/L + 2.0 mg/L IBA

T25= BA 3.0 mg/L + 2.5 mg/L IBA

The experiments were arranged in Completely Randomized Design (CRD) with five replications. Each of replications consisted of five culture vials.

3.5.4 Sub-experiment - 4

Acclimatization and establishment of plantlets on soil

Tissue culture derived plantlets were acclimatized in shade house and natural condition to find out the survival percentage.

3.6 Preparation of culture media from MS powder

To prepare 1000 mL of culture media the following steps were followed:

I. 700 mL of sterile distilled water was poured into 1000 mL beaker.

II. 5 gm of MS media (readymade) and 30 gm of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

III. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

IV. The volume was made up to 1000 mL with addition of sterile distilled water.

V. The pH was adjusted at 5.8.

VI. 10 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

VII. Required volume of hot medium was dispensed into culture vials. After dispensing and proper cooling of the medium, the culture vials were plugged with cork and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.7 Steam heat sterilization of media (Autoclaving)

For sterilization the culture medium was poured in 200 mL culture vials and then autoclaving was carried out at a temperature of 121°C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving, the media were stored in at 23±2 °C for several hours to make it ready for inoculation with explants.

3.8 Sterilization of culture room and transfer area

In the beginning, the culture room was sprayed with formaldehyde and then the room was kept closed for 3 days. After that the room was cleaned through gently washing the floors, walls and rakes with detergent. This is followed by careful wiping them with 70% ethanol. This process of sterilization of culture room was repeated at regular intervals. The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar airflow cabinet was usually sterilized by switching UV ray to kills the microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiped with 70% ethanol before starting the transfer work.

3.9 Preparation of explants and sterilization

The explants (Cladodes) were washed thoroughly under running tap water and then autoclaved distilled water for several times. Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 mL sterilized beaker. The beaker with explants was constantly shaken during sterilization. They were treated with 70% ethanol for 1 minute and rinsed with autoclaved distilled water for 3-4 times. After treating with 70% ethanol, the explants were surface sterilized with a 0.1% mercuric chloride ($HgCl_2$) solution containing two-three drops of Tween-20 for 5 min under aseptic condition and then

washed 4-5 times with autoclaved distilled water to make the material free from chemicals. Young cladodes were then transferred into Petri dishes as explants are ready for inoculation in culture media.

3.10 Inoculation of explant in culture media

For inoculation, the workers hands and forearms were washed thoroughly with soap or antiseptic and repeatedly sprayed with 70% alcohol during the period of work. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with same alcohol. All glassware, instruments and media were steam sterilized in the autoclave. During the course of work, small instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. Explants were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants (Cladodes) were further trimmed and extra spines-hair were removed with sterile scalpel blade to make suitable size. The surface sterilized explants (Cladodes) were inoculated carefully following proper sterilization process within laminar airflow cabinet. The mouth of culture vial was flame sterilized before and after positioning of the explants (Cladodes) on the media. After cutting explants into suitable size (1-3 cm), explants were transferred to culture bottles containing 20 mL MS medium with plant growth regulator. After vertically inoculating the explants singly in culture vials, the mouth of vials were quickly flame sterilized again and capped tightly. After proper labeling, mentioning media code, date of inoculation etc., the vials were transferred to growth room. Some explants became black in color within 6-7 days after inoculation. To control blackening, the blackish tissues on the explants were removed and the explants were transferred to similar fresh medium. It was repeated each of 10 days interval for about one month to minimize further blackening of the tissue

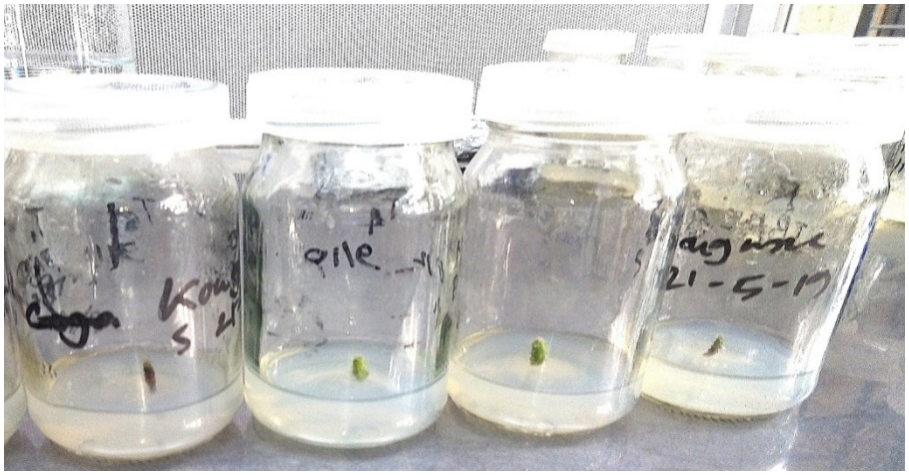


Plate 3. Inoculation of explant in culture media

3.11 Incubation

The culture vials then transferred to culture racks and allowed to grow in controlled environment. All media were prepared by standard procedures and the culture bottles were transferred to growth chamber with a photoperiod of 16 hr light and 8 hr of dark at 21 ºC ± 1 ºC with 3000-5000 lux light intensity and 70% relative humidity (R_H) incubated for 3 months.

3.12 Sub-culturing and maintaining of proliferating shoots

Initial sub-culturing was done after 15 to 30 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces. Shoots were excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency.

The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 2^{nd} weeks from inoculation, so that each piece would contain about one shoot. Blackish or brownish basal tissues were removed. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of 20-25 days.

3.13 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 IBA at each (0.5, 1.0, 1.5, 2.0 and 2.5 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 1.06 kg/cm^2 (15 PSI) for 15 min.

3.14 Transfer of plantlets from culture vials to soil

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 and then finally in field condition.

3.15 Data recording

The observations on development pattern of shoots and roots were made throughout the entire culture period. Three replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 2, 3 and 4 weeks of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week to fifth week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Days to shoot induction
2. No. of shoots per explants
3. Average length of shoot (cm)
4. Days to root induction
5. No. of roots per explants
6. Average length of root (cm)
7. Percent of explants showing shoot induction
8. Percent of explants showing root induction
9. Survival rate

3.15.1 Calculation of percentage of shoot induction

Days to shoot and root induction were calculated by counting the days from explants inoculation to the first induction of shoot.

$$\text{Percentage of shoot induction} = \frac{\text{Number of explants induced shoots}}{\text{Number of explants inoculated}} \times 100$$

3.15.2 Days to shoot induction

Days to shoot induction were calculated by counting the days from explants inoculation to the first induction of shoots.

3.15.3 Number of shoots per explant

Number of shoots per explant was calculated by using the following formula,

$$\text{Number of shoots per explant} = \frac{\text{Number of shoots per explant}}{\text{Number of observations}} \times 100$$

3.15.4 Percent of explants showing root induction

The number of roots produced per explant were recorded and the percentage of root regeneration was calculated as number of days required for initiation of root from the day of inoculation was recorded.

$$\text{Percentage of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot inoculated}} \times 100$$

3.15.5 Number of roots per plantlet

Average number of roots/plantlet was calculated by using formula.

$$\text{Number of roots per explant} = \frac{\text{Number of roots per explant}}{\text{Number of observations}} \times 100$$

3.15.6 Length of roots

Root length was determined in centimeter (cm) from the base to tip of the roots. Average length of the root was calculated by using formula.

3.15.7 Percentage of established plantlets

The percentages of established plantlets were calculated based on the number of plantlets placed in the plastic pots and the number of plants finally survived. The percentages of established plantlet were calculated by using the following formula:

$$\text{Percentage of established plantlets} = \frac{\text{Number of established plantlets}}{\text{Number of plantlets inoculated}} \times 100$$

3.15.8 Calculation of number of shoots and roots per plant

Number of shoot and root per explants was calculated by using the following formula:

$$\text{Number of shoot/roots per plant} = \frac{\text{Number of shoots/roots per explant}}{\text{Number of observations}} \times 100$$

3.15.9 Calculation of shoot and root length (cm)

Shoot and root length were measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the means were calculated.

3.15.10 Calculation of survival rate of plantlets

The survival rate of established plants was calculated based on the number of plantlets placed in the pot and the number of plants finally established or survived by the following equation,

$$\text{Survival rate of plantlets} = \frac{\text{Number of established plantlets}}{\text{Total number of plantlets}} \times 100$$

3.16 Statistical data analysis

Data recorded for different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The means for all the treatments were calculated and analyses of variance of all the characters were performed. Experiment was conducted in laboratory and arranged in Completely Randomized Design (CRD) with three replications. The significant difference between the pair of means was evaluated by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

CHAPTER - IV

RESULT AND DISCUSSION

CHAPTER IV
RESULT AND DISCUSSION

Different investigations were made on this experiment under laboratory condition to evaluate the effect of different plant growth regulators on multiple shoot and root induction in Cactus. The overall objective of the present study has been to develop a system for the mass propagation of Cactus. Optimising the relative ratio of different growth regulators has been successfully used in the current investigation. The results of these experiments have been presented and discussed in this chapter with Plates (4-12), Figures (1-8) and Tables (1-8). Analyses of variance in respect of all the parameters have been presented in Appendices (II-XXXI).

4.1 Sub-experiment 1. Effect of BA on multiple shoot proliferation Cactus (*Opuntia monacantha*)

This experiment was conducted under laboratory condition to evaluate the effect of different plant growth regulators on multi shoot proliferation. Optimising the relative ratio of auxin to cytokinin has been successfully used in the current investigation. The response of explant to different plant growth regulators singly or in combination varied significantly. The results are presented separately under different headings below. The results of the effect of different concentrations of BA have been presented under following headings with Figures 1-2, Tables 1-2 and Plates 4-5.

4.1.1 Percentage of explant showing shoot induction

There was significant variation on the percentage of explant showing shoot induction at different concentrations level of BA. The highest percentage (93.64%) of shoot induction was induced in treatment 2.5 mg/L BA and the lowest percentage (32.5%) was induced in hormone free media. On the contrary 43.72% ,55.96% ,66.44% and 69.76% shoot induction were observed respectively from 1.0 mg/L, 1.5 mg/L, 2.0 mg/L and 3.0 mg/L BA contained media (Figure 1). Batista *et al*. (2018) found the highest 93.3 % shoot

induction with 12.5 µM BA in Cactus. Ruvalcaba-Ruiz *et al*. (2014) reported the highest 89.6 % shoot induction with 15 µM BA in Cactus. The findings of this study are quite similar with their results.

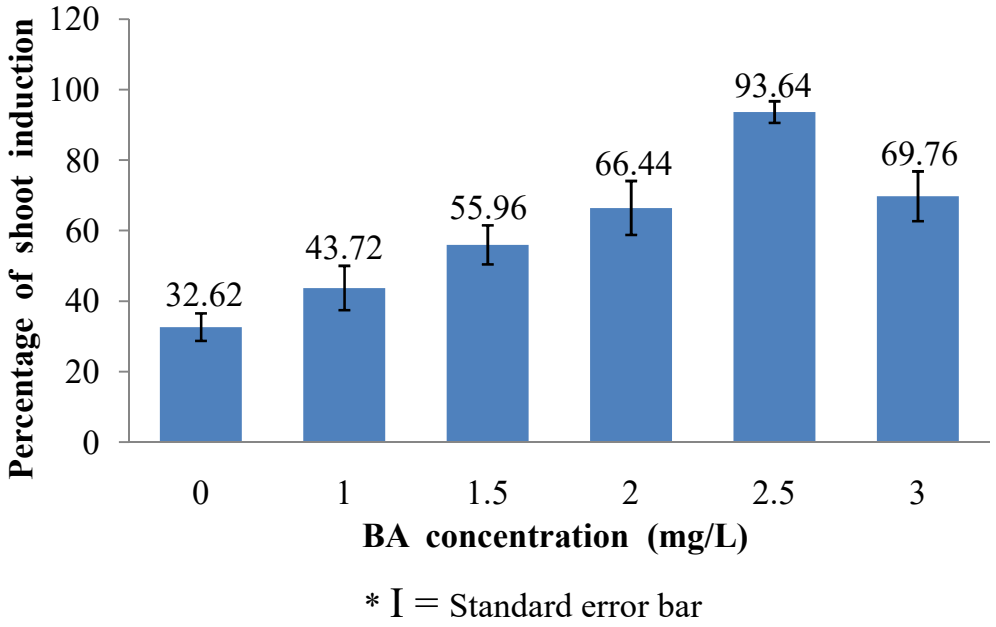


Figure 1. Percentage of shoot induction at different concentration of BA in Cactus

4.1.2 Days to shoot induction

Significant variations were observed among different concentration of BA on days to shoot induction. The maximum 20.6 days to shoot induction were recorded in control treatment followed by the treatment 1.0 mg/L, 1.5 mg/L, 2.0 mg/L and 3.0 mg/L of BA contained media required 17.6 days, 13.4 days, 10.8 days and 10.2 days respectively. On the contrary, minimum 5.4 days was required in the treatment 2.5 mg/L of BA contained media (Figure 2). The findings of this study showed consistency with the results of Rosa-Carrillo *et al*. (2012) where he reported that 29 days were required to induce shoot without any BA in the media and found minimum 5.4 days to induce shoot with 2.5 mg/L BA supplemented media. Again, Joseph *et al*. (1998) reported the minimum 5.6 days to induce shoot with BA contained media.

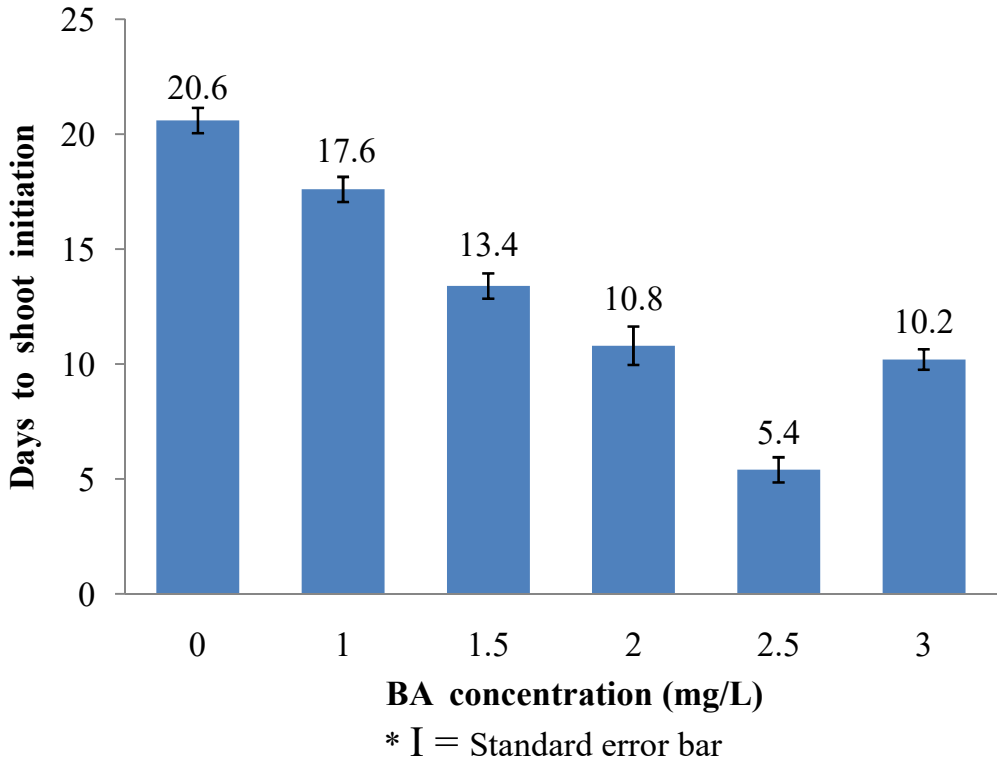


Figure 2. Days to shoot induction in Cactus with different concentration of BA

4.1.3 Number of shoots per explant

There was a significant variation of different concentrations of BA on the number of shoots per explant at 5% level of significance. The treatment 2.5 mg/L BA gave the highest number of shoots (4.4, 5.4 and 6.6) at 2 WAI, 3 WAI and 4 WAI, respectively whereas the lowest number of shoots (0.0 , 1.0 and 1.0) at 2 WAI, 3 WAI and 4 WAI, respectively were found with hormone free media. On the contrary, BA concentration of 1 mg/L gave shoots (0.0, 1.4 and 1.6) at 2 WAI, 3 WAI and 4 WAI, respectively; BA concentration of 1.5 mg/L gave shoots (1.4, 1.8 and 2.8) at 2 WAI, 3 WAI and 4 WAI, respectively; BA concentration of 2 mg/L gave shoots (2.0, 3.0 and 3.6) at 2 WAI, 3 WAI and 4 WAI, respectively and BA concentration of 3 mg/L gave shoots (1.8, 2.4 and 3.6) at 2 WAI, 3 WAI and 4 WAI, respectively (Figure 3, Table 1 and Plate 4). Lizalde *et al*. (2003) observed 6.5 shoots per explant in MS media suplimented with 2.5 mg/L BA. Sanchez-Moran and Perez-Molphe-Balch (2007) reported that the highest 6.0 shoots per explants in MS media contained 2.3 mg/L of BA. Vinas *et al*. (2012) observed 1.0 shoots per explant

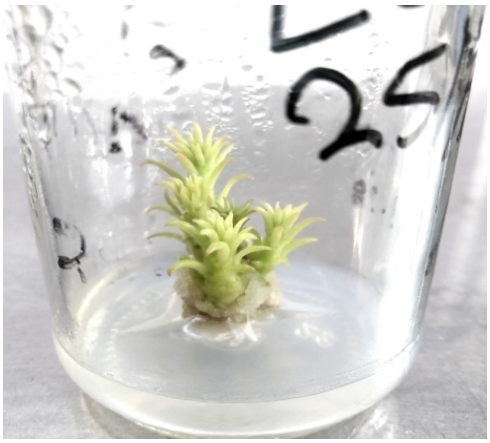
in media supplemented with 7.2 mg/L BA in Cactus. The number of shoots produced by tissue culture techniques can vary according to plant genotype and explant type, as well as concentration and nature of plant growth regulators (PGRs) used (Rocha *et al*., 2014, Kulus, 2015 and Krishna *et al*., 2016).

Table 1. Effect of different concentration of BA on number of shoot in Cactus at different WAI

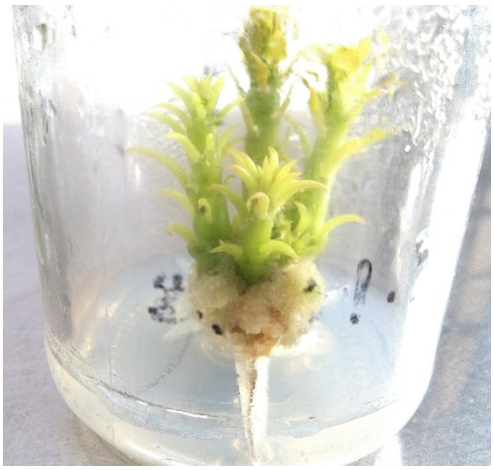
BA (mg/L)	Number of shoot/explant		
	2 WAI	3 WAI	4 WAI
0.0	0.2 c	1.0 c	1.0 d
1.0	0.2 de	1.4 de	1.6 d
1.5	1.4 cd	1.8 cd	2.8 c
2.0	2.0 b	3.0 b	3.6 b
2.5	4.4 a	5.4 a	6.6 a
3.0	1.8 bc	2.4 bc	3.6 b
CV(%)	5.09	5.7	4.19
LSD(0.05)	0.7150	0.6741	0.6305

* WAI=Weeks After Inoculation, CV = Coefficient of variation, LSD = Least significant difference (Different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly)

A



B



C

Plate 4. Shoot proliferation of Cactus on MS media supplemented with 2.5 mg/L BA (A) Induction of shoot in 2 WAI (B) Induction of shoot in 3 WAI (C) Induction of shoot in 4 WAI

4.1.4 Length of shoots per explants (cm)

Significant variations was observed in different concentrations of BA on average length of shoots were found at 5% level of significance. Data were recorded after 2, 3 and 4 weeks of culture on MS media. The optimum length of shoot (2.58 cm, 5.1 cm and 7.18 cm) at 2, 3 and 4 WAI, respectively was observed from the 2.5 mg/L BA (Plate 5 and Table 2) which was statistically different from the rest of the treatments. Whereas, minimum shoot length (0.1 cm, 0.11 cm and 0.15 cm) at 2, 3 and 4 WAI respectively were recorded in control treatment. Escobar *et al*. (1986) reported that the highest length of shoot (7 cm) was found in Cactus by using 2.5 mg/L of BA. Ault *et al*. (1987) also reported that the highest length of shoot (7 cm) was found in media supplemented with 2.5 mg/L BA. The findings of this study are quite similar with their results.

5A **5B**

Plate 5. The highest length of shoot (A) at 3 WAI and (B) 4 WAI with the treatment of 2.5 mg/L of BA

Table 2. Effect of different concentration of BA on Length of shoot per explant at different WAI

BA (mg/L)	Length of shoot/explant		
	2 WAI	3 WAI	4WAI
0.0	0.10 e	0.11 e	0.154 f
1.0	0.10 e	0.184 e	0.276 e
1.5	0.27 d	0.58 d	1.05 d
2.0	0.66 c	1.30 c	1.96 c
2.5	2.58 a	5.10 a	7.18 a
3.0	0.82 b	1.55 b	2.30 b
CV(%)	7.97	4.49	3.78
LSD(0.05)	0.0749	0.0861	0.1064

*WAI= Weeks After Inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

4.2 Sub-experiment 2. Effect of IBA on root induction potentiality

To develop root, the cladodes were excised from mother plant and transferred to rooting media supplemented with IBA . The results of the experiment have been presented under different heading utilizing Figures 3-5, Table 3 and Plates 6-7.

4.2.1 Percent of explant showing root induction

There was considerable variation among the different concentration of IBA on the percent of explant showing root induction (Figure 4). The highest percentage (93.8%) of root induction was recorded with 2.0 mg/L IBA. The lowest percentage (38.9%) of root induction was recorded in control treatment. With IBA, 21 species of Mexican Cacti were rooted (Perez-Molphe-Balch *et al*., 1998). Again satisfactory rooting occurred for three *Opuntia* genotypes treated with IBA (Garcia-Saucedo *et al*., 2005). Moreover 100% of shoots rooted after six weeks of culture for a Moroccan Cacti (El Finti *et al*., 2012). Rooting of several *Opuntia* species is favored in presence or absence of auxins in culture media (Hartmann *et al*., 1997). Maria *et al*. (2005) reported that

92.8% root induction were observed in media supplemented with 2.0 gm/L IBA.

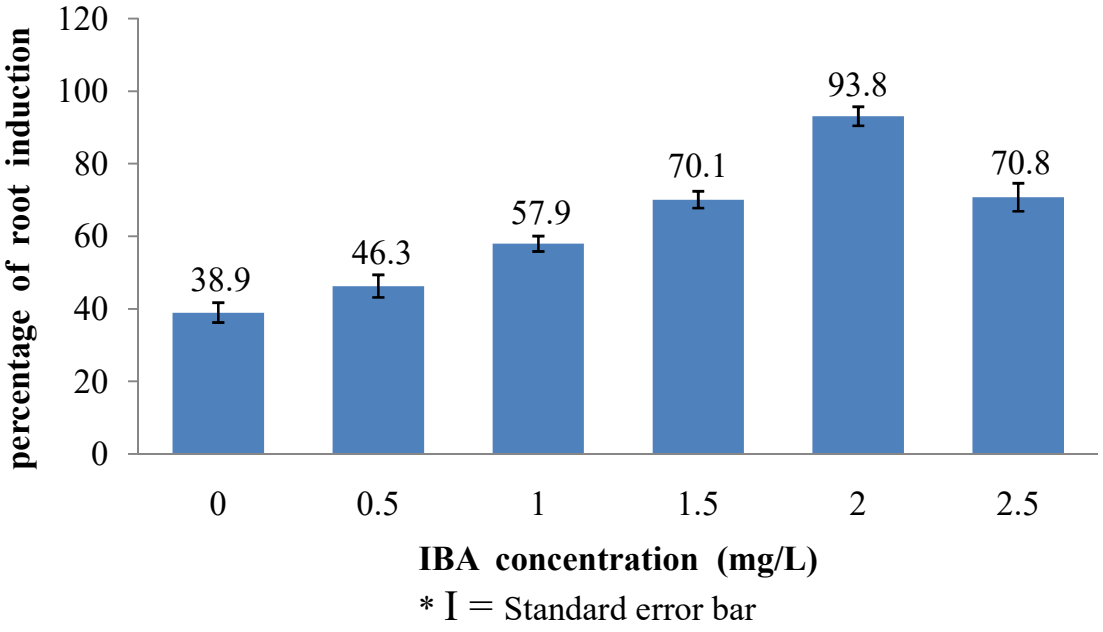


Figure 3. Percentage of root induction with different concentration of IBA in Cactus

4.2.2 Days to root induction

Significant variations were observed among different concentrations of IBA on days to root induction. The maximum 20.6 days to root induction were recorded in control treatment followed by 0.5 mg/L IBA (16.6 days), 1 mg/L (13.6 days) and 1.5 mg/L (11.6 days). On the contrary, minimum 5.4 days were recorded in 2.0 mg/L IBA followed by 10.80 days were recorded in 2.5 mg/L IBA (Figure 5). Khalafalla *et al.* (2007) reported that the minimum root induction occurred in 6 days in media contained with 1.8 mg/L IBA. Lizalde *et al.* (2003) reported that the minimum 5.8 days to root induction was observed in 15 µM IBA supplemented media. Juarezi *et al.* (2003) also reported that almost all *Opuntia* species induce root in minimum days with 1.5 mg/L IBA to 2.5 mg/L IBA supplemented media. This variation may be due to growth regulators in the culture media, genetic, physiological and morphological change in *in vitro* (Chaturvedi *et al.*, 2007).

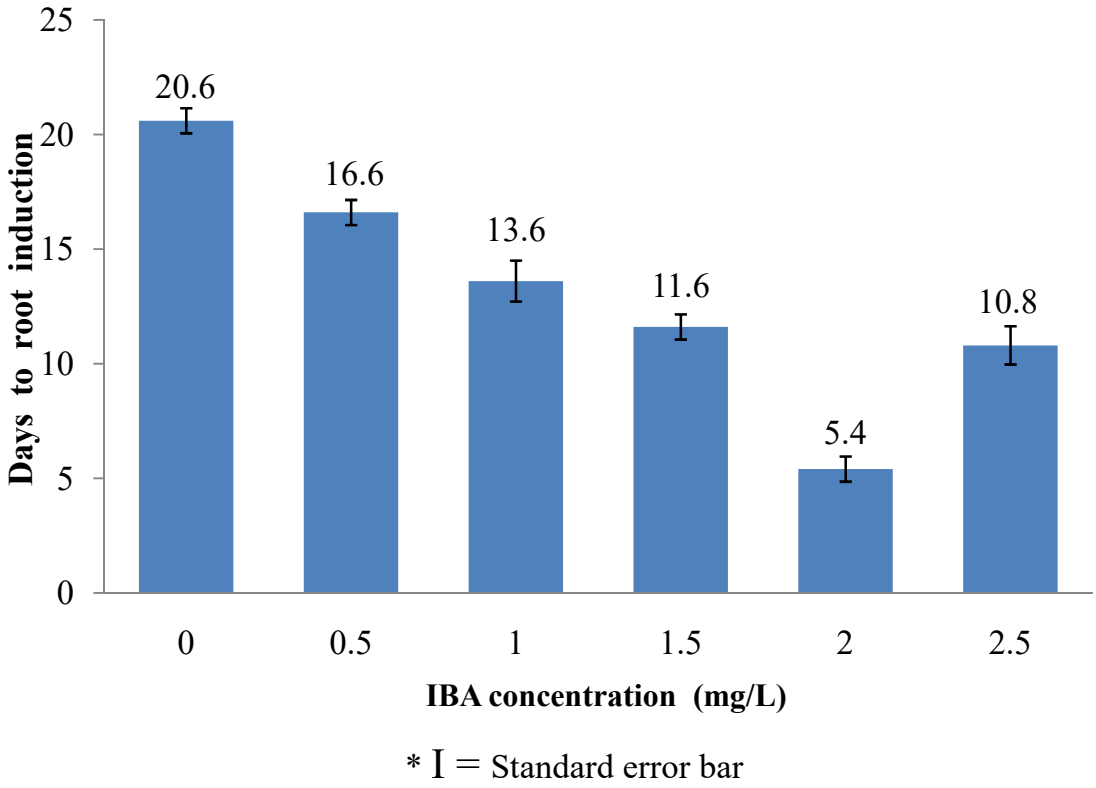


Figure 4. Days to root induction in Cactus with different concentration of IBA

4.2.3 Number of roots per explant

There was significant variation of different concentrations of IBA on the number of roots per plantlet. IBA 2.0 mg/L gave the highest number of root (4.8, 6.4 and 6.8) at 2 WAI, 3 WAI and 4 WAI, respectively and the control treatment showed the lowest number of root (0.2 , 1.0 and 1.4) at 2 WAI, 3 WAI and 4 WAI, respectively (Plate 6, Figure 6 and Table 3). Although the number of roots produced by IAA is lower than the IBA, this number remains important in comparison with the results of other studies where the medium was free hormones (Davila-Figueroa *et al*., 2005). These results affirmed that using IBA allowed shoots to form roots at greatest number (Escobar-Araya *et al*., 1986, Garcia-Saucedo *et al*., 2005 and Mohamed-Yasseen *et al*., 1995). Furthermore, Garcia-Saucedo *et al*. (2005) indicated that IBA interacted significantly with the culture medium and the materials, having a strong influence for plantlet rooting.

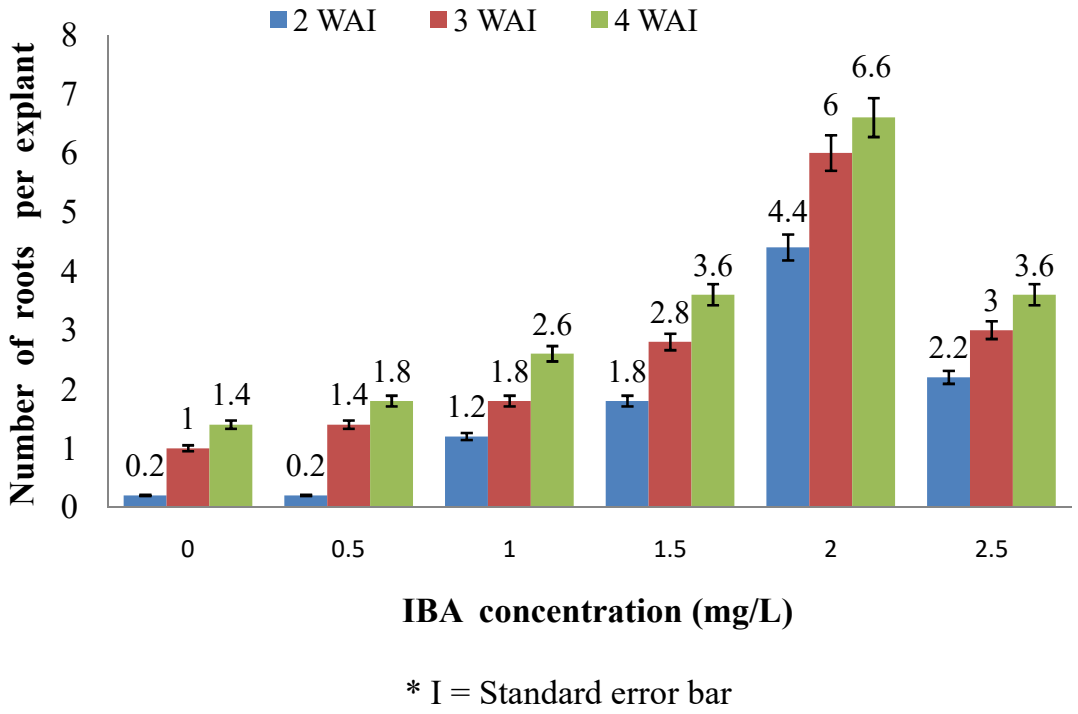


Figure 5. Number of roots in Cactus with different concentration of IBA

A **B**

Plate 6. Number of roots development (A) view through the body (B) view through the base in Cactus with the treatment of 2.0 mg/L IBA

4.2.4 Length of roots per explant (cm)

There was a significant influence of different concentrations of IBA on the length of root at 5% level of significance. The highest length of roots (3.28 cm, 5.42 cm and 7.20 cm) were recorded at 2 WAI, 3 WAI and 4 WAI, respectively (Plate 7 and Table 4) was observed from the 2.0 mg/L IBA. Then 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.5 mg/L of IBA contained media showed (0.03 cm, 0.2 cm and 0.34 cm) , (0.6 cm , 0.88 cm and 1.20 cm), (1.07 cm, 1.59 cm and 2.03 cm) and (1.38 cm, 1.86 cm and 2.45 cm) length of root at 2 WAI,3 WAI and 4 WAI, respectively. On the contrary, the lowest length of root (0.0 cm, 0.1 and 0.2 cm) at 2 WAI, 3 WAI and 4 WAI, respectively was observed in control treatment. Landsmann *et al*. (1999) reported that the highest root length (7.15 cm) of *Opuntia fiscus* was found with 12 µM IBA supplemented media. Anthony *et al*. (1991) reported that the minimum root length of *Opuntia* species rages from 2.3 cm to 7 cm with 2 mg/L IBA. Again El Finti *et al*. (2012) reported that the Moroccan Prickly Pear Cactus gave root to grow up in the length ranged between 2.19 to 6.93 cm with a significant difference between these values. The findings of this study are almost similar with their results.

Plate 7. Length of root at 4 WAI with the treatment of 2.00 mg/L IBA

Table 3. Effect of different concentration of IBA on Length of root per explant at different WAI

BA (mg/L)	Length of root/explant		
	2 WAI	3 WAI	4 WAI
0.0	0.02 e	0.104 e	0.20 e
0.5	0.03 e	0.20 e	0.34 e
1.0	0.60 d	0.88 d	1.20 d
1.5	1.07 c	1.59 c	2.03 c
2.0	3.28 a	5.42 a	7.20 a
2.5	1.38 b	1.86 b	2.45 b
CV(%)	10.04	7.99	5.81
LSD(0.05)	0.1390	0.1747	0.1697

*WAI=Weeks After Inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

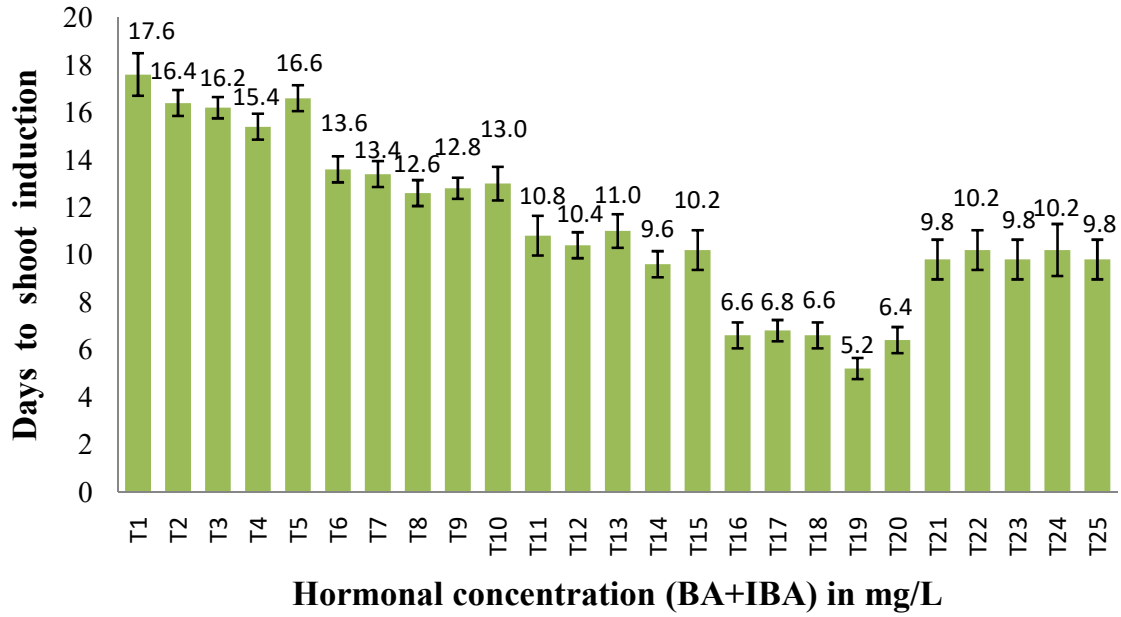
4.3 Sub-experiment 3. Combine effect of BA and IBA on shoot and root induction potentiality in Cactus (*Opuntia monacantha*)

The results of the combined effect of different concentrations of BA and IBA have been presented under following headings with Figures 6-8, Tables 4-7 and Plates 8-11.

4.3.1 Days to shoot initiation

Significant variations were observed for the different concentrations of BA and IBA on days to shoot induction. The minimum duration 5.2 days was obtained in BA (2.5 mg/L) + IBA (2.0 mg/L) than rest of the treatments. On the contrary, the maximum days (17.6 days) to shoot induction was recorded in BA (1.0 mg/L) + IBA (0.5 mg/L) (figure 6). Machado and Prioli (1996) reported that the minimum 6.1 days were required to induce shoot with BA (4.4 µM) + IAA (5.71 µM). Davila-Figueroa *et al*. (2005) reported that minimum 6.5 days

were required to induce shoot with BA (2.3 mg/L) + IBA (2.1 mg/L) contained media. Anthony *et al.* (2002) found that in *Coryphantha elephantidens* the minimum 7 days and maximum 34 days were required to initiate shoot with BA (8.9 µM) + NAA (0.54 µM) contained media. This variations may be occurred due to the age, nature, origin and the physiological state of the explants and PGRs play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj *et al*., 1995).



* I = Standard error bar

Hormonal concentration in mg/L (BA+IBA)

Treatments (BA+IBA in mg/L)				
T1=1.0+0.5	T2= 1.0+1.0	T3=1.0+1.5	T4=1.0+2.0	T5=1.0+2.5
T6=1.5+0.5	T7=1.5+1.0	T8=1.5+1.5	T9=1.5+2.0	T10=1.5+2.5
T11=2.0+0.5	T12=2.0+1.0	T13=2.0+1.5	T14=2.0+2.0	T15=2.0+2.5
T16=2.5+0.5	T17=2.5+1.0	T18=2.5+1.5	T19=2.5+2.0	T20=2.5+2.5
T21=3.0+0.5	T22=3.0+1.0	T23=3.0+1.5	T24=3.0+2.0	T25=3.0+2.5

Figure 6. Days to shoot induction in Cactus at different concentration of BA and IBA

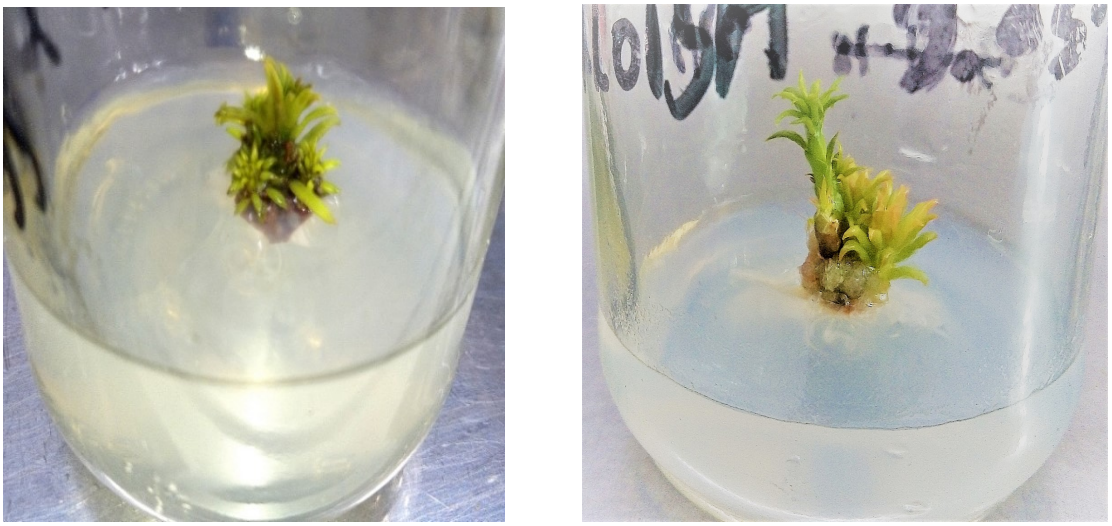
4.3.2 Percentage of shoot initiation

There was a significant influence of different concentrations of BA and IBA on the percentage of shoot induction per explant. The maximum percentage (94.6%) of shoot induction was recorded in BA (2.5 mg/L) + IBA (2.0 mg/L) which was among other treatments and minimum percentage (43.4%) was recorded in BA (1.0 mg/L) + IBA (0.5 mg/L) (Table 4). Perez-Molphe-Balch *et al*. (2002) reported that maximum percentage (91%) of shoot initiation was observed with BA (10 μM) + IBA (4.9 μM) contained media. Malda *et al*. (1999) reported that maximum percentage (94.5%) of shoot induction was recorded with BA (2.5 mg/L) + IBA (2.0 mg/L) supplemented media. The findings of this study are comparable with their results.

4.3.3 Number of shoots per plantlet

Different concentrations of BA and IBA showed significant variations on the number of shoot at 5% level of significance. The maximum number of shoots (4.2, 5.2 and 7.0) were recorded from the BA (2.5 mg/L) + IBA (2.0 mg/L) at 2 WAI, 3 WAI and 4 WAI respectively. Then the second highest number (3.4, 4.4 and 5.8) was noticed at 2 WAI, 3 WAI and 4 WAI, respectively, were observed in BA (2.5 mg/L) + IBA (1.5 mg/L). On contrary, the lowest number of shoot (0.2, 1.0 and 1.4) were observed at 2 WAI, 3 WAI and 4 WAI, respectively in BA (1.0 mg/L) + IBA (0.5 mg/L) (Plate 8 and Table 4). Infante (1992) reported that the maximum number of shoots (3.65, 4.1 and 6.05) were observed with BA (2.4 mg/L) + IBA (1.8 mg/L). Johnson and Emino (1979) reported that the highest number of shoots (2.4, 3.7 and 5.8) were found with BA (13 μM) + IBA (9 μM) contained media. The findings of this study are in consistent with those results. Shoot proliferation responses varied according to the type of PGRs and concentration added to the culture medium (Estrada-Luna *et al*., 2008).

Various PGRs have been adopted both for culture initiation and shoot proliferation of the Prickly pear (Juarez and Passera, 2002, Khalafalla *et al*., 2007 and Mohamed-Yasseen *et al*., 1995).



A **B**

Plate 8. Combine effect of BA + IBA (2.5 mg/L+ 2 mg/L) on the number of shoot induction at (A) 2 WAI (B) 3 WAI

4.3.4 Length of shoots (cm)

There was a significant variation in different concentrations of BA with IBA on the length of shoot at 5% level of significance. The highest length of shoot (2.53 cm, 5.04 cm and 6.78 cm) at 2 WAI, 3 WAI and 4 WAI, respectively were recorded from the BA (2.5 mg/L) + IBA (2.0 mg/L) . On the contrary, the minimum length of shoot (0.1 cm, 0.172 cm and 0.242 cm) at 2 WAI, 3WAI and 4WAI, respectively were observed in BA (1.0 mg/L) + IBA (0.5 mg/L) (Table 5 and Plate 9). Rosa Carrillo *et al*. (2012) reported that the highest length of shoot (2.5 cm, 5.3 cm and 6.52 cm) were observed in BA (2.5 mg/L) + IBA (2.0 mg/L) contained MS media. Vyskot and Jara (1984) reported that

the maximum length of shoot (2.6 cm , 5.02 cm and 6.8 cm) were observed in BA (2.5 mg/L) + IBA (2.0 mg/L) contained MS media. The findings of this study are almost similar to the results of those researchers.

Plate 9. The highest length of shoot at 4 WAI with the treatment of BA+ IBA (2.5 mg/L + 2.0 mg/L)

Table 4. Combine effect of different concentration of BA and IBA on days to shoot induction potentiality

BA (mg/L)	IBA (mg/L)	Shoot induction potentiality			
		Percentage of shoot induction	Number of shoots/explant		
			2 WAI	3 WAI	4 WAI
1.0	0.5	43.4	0.1 h	1.0 f	1.4 i
	1.0	41.6	0.2 h	1.2 f	1.8 hi
	1.5	45.5	0.1 h	1.2 f	1.6 i
	2.0	49.8	0.1 h	1.2 f	1.4 i
	2.5	47.3	0.2 h	1.2 f	1.6 i
1.5	0.5	55.3	1.2 fg	1.4 ef	2.0 g-i
	1.0	54.5	1.0 g	1.4 ef	2.6 e-g
	1.5	58.9	1.4 e-g	2.2 cd	2.6 e-g
	2.0	59.5	1.2 fg	2.2 cd	2.4 f-h
	2.5	55.8	1.4 e-g	2.2 cd	2.6 e-g
2.0	0.5	65.5	1.6 ef	2.6 cd	3.2 c-e
	1.0	61.2	1.8 de	2.2 cd	2.6 e-g
	1.5	64.5	2.2 d	2.8 c	3.4 cd
	2.0	69.7	1.6 ef	2.6 cd	3.2 c-e
	2.5	68.8	1.8 de	2.8 c	2.8 d-f
2.5	0.5	87.6	2.8 c	4.0 b	5.2 b
	1.0	88.9	3.2 bc	4.4 b	5.6 b
	1.5	90.1	3.4 b	4.4 b	5.8 b
	2.0	94.6	4.2 a	5.2 a	7.0 a
	2.5	89.6	3.4 b	4.4 b	5.8 b
3.0	0.5	68.1	1.6 ef	2.6 cd	3.8 c
	1.0	63.7	1.6 ef	2.6 cd	3.4 cd
	1.5	65.4	1.8 de	2.2 cd	3.0 d-f
	2.0	69.8	1.6 ef	2.0 de	3.0 d-f
	2.5	67.7	2.2 d	2.8 c	3.4 cd
CV(%)		-	12.81	7.60	4.86
LSD(0.05)		-	0.5723	0.6544	0.6873

* WAI = Weeks After Inoculation. Figures in a column followed by no letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05) = Least significant difference.

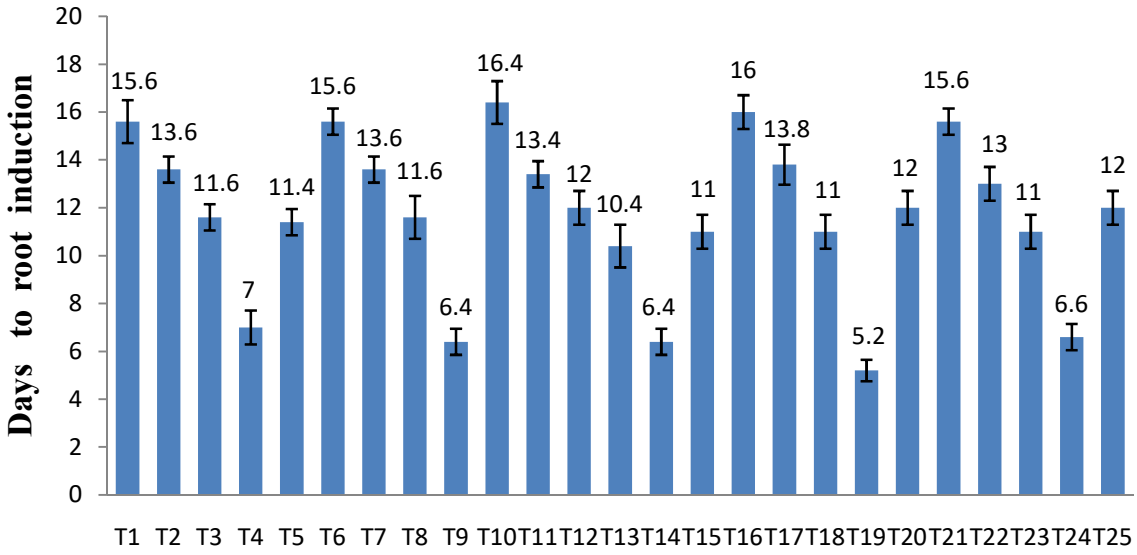
Table 5. Combine effect of different concentration of BA and IBA on the length of the shoots per explants

BA (mg/L)	IBA (mg/L)	Length of shoots/explant		
		2 WAI	3 WAI	4 WAI
1.0	0.5	0.0 n	0.172 n	0.242 o
	1.0	0.0 n	0.154 n	0.207 o
	1.5	0.0 n	0.202 n	0.306 n
	2.0	0.0 n	0.176 n	0.266 no
	2.5	0.0 n	0.154 n	0.250 no
1.5	0.5	0.246 l	0.450 m	0.916 k
	1.0	0.196 m	0.444 m	0.786 lm
	1.5	0.280 l	0.544 l	0.826 l
	2.0	0.400 k	0.700 k	0.950 k
	2.5	0.250 l	0.550 l	0.750 m
2.0	0.5	0.750 h	1.242 j	1.846 j
	1.0	0.650 i	1.328 i	1.950 i
	1.5	0.650 i	1.294 ij	1.788 j
	2.0	0.768 gh	1.226 j	2.044 h
	2.5	0.560 j	1.410 h	2.086 h
2.5	0.5	2.030 b	4.060 b	6.560 b
	1.0	1.960 c	3.862 d	6.382 d
	1.5	1.908 d	3.940 c	6.450 c
	2.0	2.530 a	5.040 a	6.788 a
	2.5	1.850 e	3.848 d	6.298 e
3.0	0.5	0.780 gh	1.470 gh	2.102 h
	1.0	0.800 g	1.512 fg	2.242 g
	1.5	0.750 h	1.550 f	2.362 f
	2.0	0.896 f	1.556 f	2.356 f
	2.5	0.850 f	1.650 e	2.376 f
CV(%)		5.07	3.77	2.00
LSD(0.05)		0.0486	0.0729	0.0595

* WAI = Weeks After Inoculation. Figures in a column followed by no letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05) = Least significant difference.

4.3.5 Days to root induction

Significant variations were observed among different concentrations of BA and IBA on days to root induction. The maximum days (16.4) to root induction was recorded in BA (1.5 mg/L) + (2.5 mg/L) IBA and minimum days (5.2) were observed in BA 2.5 mg/L + IBA 2.0 mg/L concentration (Figure 7). Khalafalla *et al*. (2007) reported that the minimum 5.6 days was observed to induced root in *Opuntia fiscus* species with BA (2.4 mg/L) + IBA (1.8 mg/L). Garcia-Saucedo *et al*. (2005) reported that the minimum 6 days were observed in Opnuntia species with BA (15 µM) + IBA (8 µM). The findings of this study are quite similar to the results of them.



* I = Standard error bar

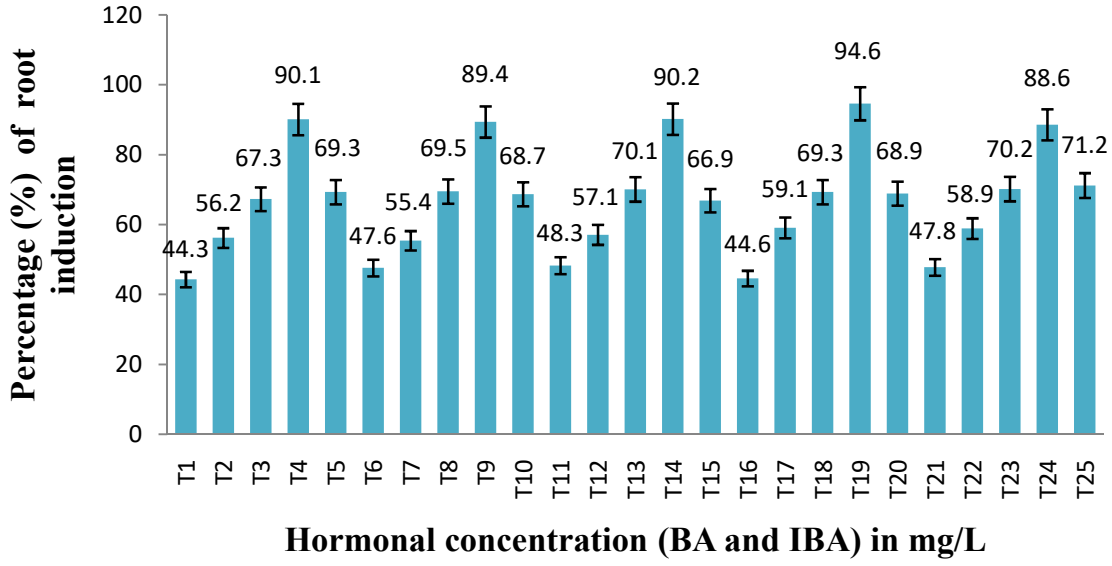
Hormonal concentration in mg/L(BA+IBA)

Treatments (BA+IBA in mg/L)				
T1=1.0+0.5	T2= 1.0+1.0	T3=1.0+1.5	T4=1.0+2.0	T5=1.0+2.5
T6=1.5+0.5	T7=1.5+1.0	T8=1.5+1.5	T9=1.5+2.0	T10=1.5+2.5
T11=2.0+0.5	T12=2.0+1.0	T13=2.0+1.5	T14=2.0+2.0	T15=2.0+2.5
T16=2.5+0.5	T17=2.5+1.0	T18=2.5+1.5	T19=2.5+2.0	T20=2.5+2.5
T21=3.0+0.5	T22=3.0+1.0	T23=3.0+1.5	T24=3.0+2.0	T25=3.0+2.5

Figure 7. Days to root induction in Cactus at different concentration of BA and IBA

4.3.6 Percentage of root induction

Different concentrations of BA and IBA showed significant variations on the percentage of root induction. The highest percentage (94.60%) of root induction was recorded in BA (2.5 mg/L) + IBA (2.0 mg/L) whereas the lower percentage (44.30%) of root induction was recorded in BA (1.0 mg/L) + IBA (0.5 mg/L) supplemented media (Figure 8). El Finti *et al*. (2012) reported that the optimum percentage (96%) of root induction was observed in BA (2.5 mg/L) + IBA (2.0 mg/L). Myeong *et al*. (2004) reported that the maximum percentage (92%) of root induction was observed in BA (2.5 mg/L) + IBA (1.8 mg/L).



* I = Standard error bar

Hormonal concentration in mg/L(BA+IBA)

Treatments (BA+IBA in mg/L)				
T1=1.0+0.5	T2= 1.0+1.0	T3=1.0+1.5	T4=1.0+2.0	T5=1.0+2.5
T6=1.5+0.5	T7=1.5+1.0	T8=1.5+1.5	T9=1.5+2.0	T10=1.5+2.5
T11=2.0+0.5	T12=2.0+1.0	T13=2.0+1.5	T14=2.0+2.0	T15=2.0+2.5
T16=2.5+0.5	T17=2.5+1.0	T18=2.5+1.5	T19=2.5+2.0	T20=2.5+2.5
T21=3.0+0.5	T22=3.0+1.0	T23=3.0+1.5	T24=3.0+2.0	T25=3.0+2.5

Figure 8. Percentage to root induction in Cactus at different concentration of BA and IBA

4.3.7 Number of roots per plantlet

There was a significant influence of different concentrations of BA and IBA on the number of roots per explant. The treatment BA (2.5 mg/L) + IBA (2.0 mg/L) gave the maximum number of roots (4.8, 6.2 and 6.6) at 2 WAI, 3WAI and 4 WAI, respectively. On the contrary, the lowest number of roots (0.1 ,1.0 and 1.4) at 2 WAI, 3 WAI and 4 WAI, was found in BA (1.0 mg/L) + IBA (0.5 mg/L) IBA supplemented media (Plate 10 and Table 6). Papafotiou *et al*. (2001) reported that the maximum number of roots (4.4, 5.1 and 7.0) were observed in BA (2.4 mg/L) + IBA (2.1 mg/L) contained media. Vyskot and Jara (1984) reported that the optimum number of roots (4.3, 6.1 and 6.5) were observed in BA (2.5 mg/L) + IBA (2.0 mg/L) contained media.

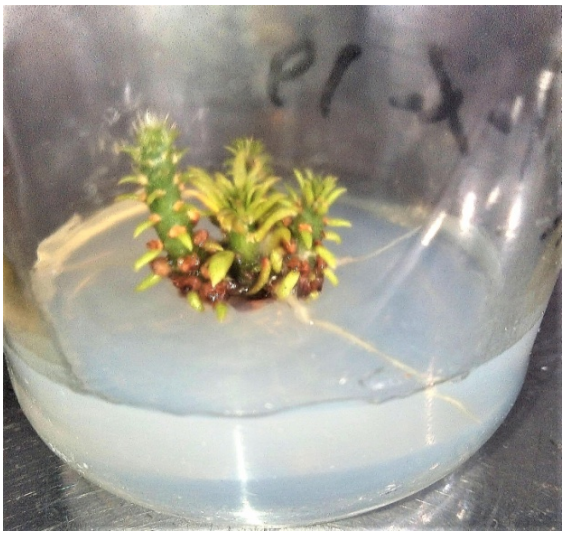


Plate 10. The highest number of root with the treatment of BA+ IBA (2.5 mg/L + 2.0 mg/L)

Table 6. Combine effect of different concentration of BA and IBA on the number of the roots per explants

BA (mg/L)	IBA (mg/L)	Root induction potentiality		
		Number of root/explant		
		2 WAI	3 WAI	4 WAI
1.0	0.5	0.1 j	1.0 h	1.4 h
	1.0	1.0 i	1.2 h	1.6 gh
	1.5	1.2 hi	2.0 fg	2.6 de
	2.0	2.8 c	3.6 c	4.4 b
	2.5	2.0 ef	2.8 de	3.6 c
1.5	0.5	0.1 j	1.2 h	1.6 gh
	1.0	1.0 i	1.4 gh	1.8 f-h
	1.5	1.4 g-i	2.2 ef	2.6 de
	2.0	2.8 c	3.8 bc	4.6 b
	2.5	1.8 e-g	2.6 d-f	2.8 de
2.0	0.5	0.1 j	1.2 h	1.4 h
	1.0	1.2 hi	1.4 gh	1.6 gh
	1.5	1.6 f-h	2.2 ef	2.2 e-g
	2.0	3.6 b	4.4 b	4.6 b
	2.5	2.2 de	2.8 de	3.2 cd
2.5	0.5	0.1 j	1.2 h	1.6 gh
	1.0	1.0 i	2.0 fg	2.4 ef
	1.5	1.8 e-g	2.4 ef	2.8 de
	2.0	4.8 a	6.2 a	6.6 a
	2.5	2.6 cd	3.2 cd	3.6 c
3.0	0.5	0.1 j	1.2 h	1.6 gh
	1.0	1.2 hi	2.0 fg	2.6 de
	1.5	1.2 hi	2.4 ef	2.6 de
	2.0	2.6 cd	4.4 b	4.6 b
	2.5	1.6 f-h	2.0 fg	3.6 c
CV(%)		6.32	5.08	4.64
LSD(0.05)		0.5204	0.7653	0.7098

*WAI=Weeks After Inoculation. Figures in a column followed by no letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.3.8 Length of root (cm)

There was significant variation of different combined concentrations of BA and IBA on the length of root at 5% level of significance. The highest length of root (3.4, 5.7 and 7.3 cm) at 2 WAI, 3 WAI and 4 WAI, respectively was observed in BA (2.5 mg/L) + IBA (2.0 mg/L). The lowest length of root was recorded in BA (1.0 mg/L) + (0.5 mg/L) IBA (0.1, 0.2 and 0.4 cm) at 2WAI, 3WAI and 4WAI, respectively (Plate 11 and Table 7). Elias-Rocha *et al*. (1998) reported that the highest length of root (3.2, 5.6 and 7.1 cm) were observed in BA (2.5 mg/L) + IBA (2.0 mg/L) contained media. El Finti *et al*. (2012) reported that the optimum length of root (3.1, 5.2 and 6.93 cm) were observed in BA (2.4 mg/L) + IBA (2.2 mg/L) contained media. The findings of this study are parallel to those results of the researchers.

A **B**

Plate 11. The longest root of Cactus (A) at 2 WAI (B) at 4 WAI obtained on MS media contained BA + IBA (2.5 mg/L+ 2.0 mg/L)

Table 7. Combine effect of different concentration of BA and IBA on the length of the roots per explants

BA (mg/L)	IBA (mg/L)	Length of roots/explants		
		2 WAI	3 WAI	4WAI
1.0	0.5	0.10 n	0.20 m	0.35 no
	1.0	0.55 l	0.75 l	0.95 m
	1.5	0.90 j	1.296 i	1.754 j
	2.0	2.10 c	4.10 c	5.50 d
	2.5	1.30 ef	1.85 ef	2.40 fg
1.5	0.5	0.00 n	0.15 m	0.25 o
	1.0	0.70 k	1.10 j	1.40 k
	1.5	1.10 hi	1.70 gh	2.10 h
	2.0	2.20 bc	4.10 c	5.60 d
	2.5	1.40 de	1.90 de	2.45 e-g
2.0	0.5	0.10 mn	0.18 m	0.35 no
	1.0	0.60 kl	0.80 l	1.15 l
	1.5	1.00 ij	1.60 h	1.95 i
	2.0	2.30 b	4.30 b	5.80 c
	2.5	1.25 fg	1.75 fg	2.35 g
2.5	0.5	0.15 m	0.25 m	0.38 n
	1.0	0.55 l	0.85 kl	1.15 l
	1.5	1.15 gh	1.80 e-g	2.15 h
	2.0	3.40 a	5.70 a	7.30 a
	2.5	1.446 d	2.00 d	2.55 e
3.0	0.5	0.00 n	0.22 m	0.45 n
	1.0	0.65 kl	0.95 k	1.45 k
	1.5	1.20 f-h	1.60 h	2.20 h
	2.0	2.10 c	4.25 b	5.95 b
	2.5	1.50 d	1.80 e-g	2.50 ef
CV(%)		7.20	6.09	3.97
LSD(0.05)		0.1436	0.138	0.1203

*WAI=Weeks After Inoculation. Figures in a column followed by no letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.4 Sub-experiment 4. Acclimatization and establishment of plantlets on soil

After 35 days of culture on rooting media, the plantlets were taken for acclimatization when a considerable shoots and roots were developed (Plate-12 Table-8). The success of transplantation of *in vitro* regenerated plants depends on a number of factors such as the rooting system, plant vigor, duration, and conditions of acclimatization etc.

Table 8. Survival rate of *in vitro* regenerated plantlets of Cactus (*Opuntia monacantha*)

Acclimatization	No. of Plantlets transplanted	Duration of observation	No. of plantlets survived	Survival rate (%)
In growth chamber	20	7 days	20	100
In shade house	20	14 days	18	90
In field condition	18	30 days	18	100

The plantlets were removed from vial carefully without any root damage. The roots were washed with running tap water for removing media from plantlets. Then, the culture vials were taken to growth cabinet for acclimatization and maintained for further observations under controlled conditions with light, temperature and relative humidity to provide a favorable condition for plant establishment. Then the *in vitro* regenerated plantlets were planted on a mixture of sand, coco peat and compost at the rate of 2:1:1. Then the plantlets transferred to shade house for acclimatization. In the shade house, plants are acclimatized and hardened before being transferred to the field conditions. At first 20 plants were transplanted and 18 (90%) were survived in shade condition. Finally in normal atmospheric condition 18 plants were transplanted among them 18 survived and survival rate was 100% (Table-8).

Inglese *et al*. (1995) found that plantlets of Cactus were successfully transferred to the soil where they grew well for 8 to 10 weeks with 100% survivability. 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 vitro regenerated plantlets reported that 100% survival when transferred to soil and there was no detectable variations among the plants with respect to morphology and growth characteristics. 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. This result is similar to the reports for other micropropagated prickly pear cactus species (Ault and Blackmon, 1987, Clayton *et al*., 1990, El Finti *et al*., 2012, Juarez and Passera, 2002 and Mohamed-Yasseen *et al*., 1995). So considering the survival rate it can be revealed that acclimatization potentiality of cactus was satisfactory.

12A **12B**

Plate 12. Acclimatization and establishment of plantlets (A) Pot in the shade condition (B) Pot in the natural condition

CHAPTER - V

SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of July 2019 to December 2019. The cladodes of Cactus were used as experimental materials in the present investigation. BA (1.0, 1.5, 2.0 , 2.5 and 3.0 mg/L) and IBA (0.5, 1.0, 1.5 , 2.0 and 2.5 mg/L) alone or in combination were used as treatment. The experiments were arranged in Completely Randomized Design (CRD) with five replications.

The treatment 2.5 mg/L BA gave the highest number of shoots (4.4, 5.4 and 6.6) at 2 WAI, 3 WAI and 4 WAI, respectively whereas the lowest number of shoots (0.1 , 1.0 and 1.0) at 2 WAI, 3 WAI and 4 WAI, respectively were recorded with hormone free media. Again the highest percentage (93.4%) of shoot induction was induced in treatment 2.5 mg/L BA and the lowest percentage (32.5%) was induced in the media which was hormone free. The highest length of shoot (2.58 cm, 5.1 cm and 7.18 cm) at 2 WAI, 3WAI and 4 WAI, respectively was observed from the 2.5 mg/L BA.

The treatment IBA 2.0 mg/L had produced the maximum percentage of root (93.8%) required minimum 5.2 days. This treatment also gave the highest number of root (4.8 , 6.4 and 6.8) at 2 WAI, 3 WAI and 4 WAI, and the control treatment gave the lowest number of root (0.2, 1.0 and 1.4) at all WAI. The maximum length of root (3.28, 5.42 and 7.20 cm) at 2 WAI, 3WAI and 4 WAI, respectively was found in 2.0 mg/L IBA and the hormone free control treatment gave the lowest length of root (0.1, 0.1 and 0.2) at all WAI.

In combine effect, the maximum percentage (94.6%) of shoot induction was observed in 5.2 days with treatment BA (2.5 mg/L) + IBA (2.0 mg/L) and the minimum percentage (43.4%) was in 17.6 days with BA (1.0 mg/L) + IBA (2.0

mg/L). The treatment BA (2.5 mg/L) + IBA (2.0 mg/L) gave the maximum number of shoots (4.2, 5.2 and 7.0) at 2 WAI, 3 WAI and 4 WAI, respectively whereas the minimum number of shoots (0.2, 1.0 and 1.4) at 2 WAI, 3 WAI and 4 WAI, respectively and were observed in BA (1.0 mg/L) + IBA (0.5 mg/L). The treatment BA (2.5 mg/L) + IBA (2.0 mg/L) gave the maximum length of shoot (2.53, 5.04 and 6.788 cm) at 2 WAI, 3 WAI and 4 WAI, respectively whereas the minimum length of shoots (0.1, 0.172 and 0.242) at 2 WAI, 3 WAI and 4WAI, respectively were observed with BA (1.0 mg/L) + (0.5 mg/L) IBA.

Again in combined effect, the maximum percentage (94.6%) of root was recorded in 5.2 days with the treatment of BA (2.5 mg/L) + IBA (2.0 mg/L) and the minimum percentage (44.30%) was in 16.4 days with the treatment of BA 1.0 mg/L + IBA 0.5 mg/L. The treatment BA (2.5 mg/L) + IBA (2.0 mg/L) gave the maximum length of root (3.4, 5.7 and 7.3 cm) at 2 WAI, 3 WAI and 4 WAI, respectively whereas the minimum length of roots (0.1, 0.2 and 0.4) at 2 WAI, 3 WAI and 4WAI, respectively were observed with the treatment of BA (1.0 mg/L) + IBA (0.5 mg/L).

Regenerated plantlets showed 100% survival in growth chamber, 90% survival during in shade conditions and 100% survival in the field condition of hardening in open atmosphere.

In case of single dose, 2.5 mg/L BA showed the best results for *in vitro* shoot regeneration and IBA 2.0 mg/L performed the best results for root induction in *in vitro* root regeneration. Overall combine dose of BA+IBA (2.5 mg/L + 2.0 mg/L) gave the best performance in *in vitro* regeneration of Cactus.

Findings of the present study showed that *in vitro* regeneration is an effective method for the rapid regeneration of Cactus. Thus the protocol of *in vitro* rapid regeneration of Cactus has been established which may contribute in large seedlings production throughout the year.

RECOMMENDATIONS

RECOMMENDATIONS

The following recommendations could be addressed based on the present experiment,

i. Other explants such as shoot tip, leaf and root tip can be experimented for the proliferation of Cactus.
ii. Further study can be done with different concentrations and combinations of auxins and cytokinines group of hormones for the rapid regeneration of Cactus.
iii. Callus induction can be done with 2,4-D or other callus induction hormone for large number of shoot induction.
iv. To understand the influence of genotype (if there is any), more research should be carried out with different types of genotypes of Cactus.

REFERENCES

REFERENCES

Akram, G., Hasanloo, T. and Nekouei, M.K. (2013). Micropropagation of tuna (*Opuntia ficus indica*) and effect of medium composition on proliferation and rooting. *Int. J. Biosci*. **3**:129-139.

Anthony, J., Smith, R.H. , Burdick, J.P. and Reilley, A.A. (1991). *In vitro* propagation of *Coryphantha macromeris*. *Int. J. Hort. Sci*. **26**(3): 315.

Ault, J.R. and Blackmon, W.J. (1987). *In vitro* propagation of *Ferocactus acanthodes*. *Hort. Sci.* **22**:126–127.

Bajaj, Y.P.S. (1995). Cryopreservation of plant cell, tissue, and organ culture for the conservation of germplasm and biodiversity. **In:** Cryopreservation of plant germplasm. Springer, Berlin, Germany. pp. 3-28.

Balen, B., Tkalec, M., Stefanic, P.P., Vidakovic-Cifrek, Z. and Krsnik-Rasol, M. (2012). *In vitro* conditions affect photosynthetic performance and crassulacean acid metabolism in *Mammillaria gracilis Pfeiff*. tissues. *Acta Physiologiae Plantarum*. **34**: 1883-1893.

Batista, D.S., Felipe, S.H.S., Silva, T.D., de Castro, K.M., Mamedes-Rodrigues, T.C., Miranda, N.A., Rios-Rios, A.M., Faria, D.V., Fortini, E.A., Chagas, K. and Torres-Silva, G. (2018). Light quality in plant tissue culture: does it matter?. *In Vitro Cell. Dev. Biol. Plant*. **54**(3):195-215.

Butera, D., Tesoriere, L., Gaudio, F., Bongiorno, A., Allegra, M., Pintaudi, A. M., Kohen, R. and Livrea, M.A. (2002). Antioxidant activities of Sicilian prickly pear (*Opuntia ficus indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin. *J. Agric. Food Chem*. **50**(23): 6895-6901.

Cardarelli, M., Borgognone, D. and Colla, G. (2010). *In vitro* propagation of *Obregonia denegrii*. *Propag. Ornam. Plants***. 10**: 29-36.

Chaturvedi, H.C., Jain M. and Kidwai, N.R. (2007). Cloning of medicinal plants through tissue culture. *A review. Ind. J. Experimental Biol.* **45**: 937-948.

Ciriminna, R., Delisi, R., Albanese, L., Meneguzzo, F. and Pagliaro, M. (2017). *Opuntia ficus indica* seed oil: Biorefinery and bioeconomy aspects. *Eur. J. Lipid Sci. Technol*. **119**(8): 1700013.

Clayton, P.W., Hubstenberger, J.F. and Phillips, G.C. (1990). Micropropagation of members of the Cactaceae Subtribe Cactinae. *J. Am. Soc. Hortic. Sci.* **115**: 337-343.

Davila-Figueroa, C.A., Rosa-Carrillo, M.L. and Perez-Molphe-Balch, E. (2005). *In vitro* propagation of eight species or sub-species of *Turbinicarpus* . *In Vitro Cell. Dev. Biol. Plant.* **41**: 540-545.

El Finti, A.R., El Boullani, F., El Ayadi, N., Ait Aabd and El Mousadik A. (2012). Micropropagation *in vitro* of *Opuntia ficus indica* in south of Morocco. *Int. j. chem. biol. sci.* **1**: 6-10.

Elias-Rocha M.A., Santos-Diaz, M.S. and Gomez, A. (1998). Propagation of *Mammillaria candida* by tissue culture techniques. *Haseltonia.* **6**: 96-101

Escobar, H.A., Villalobos, V.M. and Villegas, A. (1986). *Opuntia* micropropagation by axillary proliferation. *Plant Cell, Tissue and Organ Cult*. **7**: 269-277.

Estrada-Luna, A.A., Lopez-Peralta, C. and Cardenas-Soriano, E. (2002). *In vitro* micrografting and the histology of graft union formation of selected species of prickly pear cactus (*Opuntia spp*.). *Scientia Horticulturae*. **92**(3-4): 317-327.

Estrada-Luna, A.A., Martinez-Hernandez, J.J., Torres-Torres, M.E. and Chable-Moreno, F. (2008). *In vitro* micropropagation of the ornamental Prickly pear cactus *Opuntia lanigera* and effects of sprayed GA3 after transplantation to *ex vitro* conditions. *Sci. Hortic.* **117**: 378-385.

Gamborg, O.L., Miller, R. and Ojima, K. (1968). Nutrient requirements of suspension cultures of Soybean root cells. *Exp. Cell Res.* **50**(1): 151-158.

Garcia-Rubio, O. and Malda-Barrera, G. (2010). Micropropagation and reintroduction of the endemic *Mammillaria mathildae* to its natural habitat. *Int. J. Hortic. Sci*. **45**: 934-938.

Garcia-Saucedo, P.A., Valdez-Morales, M., Valverde, M.E., Cruz-Hernandez, A. and Paredes-Lopez, O. (2005). Plant regeneration of three *Opuntia* genotypes used as human food. *Plant Cell, Tissue and Organ Cult*. **80**: 215-219.

Giusti, P., Vitti, D., Fiocchetti, F., Colla, G., Saccardo, F. and Tucci, M. (2002). *In vitro* propagation of three endangered cactus species. *Sci. Hortic.* **95**: 319-332.

Glass, E.C. (1998). Identification guide to threatened cacti of Mexico. *Cact. Succ. J.* **1**: 206.

Gomez, K.A. and Gomez, A.A. (1984). Statistical Procedures for Agricultural Research. 2nd ed. John Wiley & Sons Inc., New York, USA, ISBN: 680

Grigson, J. (2007). Jane Grigson's Fruit Book. **In:** Prickly pear, Indian fig, Barbary fig or Tuna. S. Dickerman, (ed.). U of Nebraska Press. New York, USA. p. 380.

Guzman-Maldonado, S.H., Morales-Montelongo, A.L., Mondragon-Jacobo, C., Herrera-Hernandez, G., Guevara-Lara, F. and Reynoso-Camacho, R. (2010). Physicochemical, nutritional, and functional characters of fruits xoconostle (*Opuntia matudae*) pears from Central Mexico region. *J. Food Sci*. **75**(6): 485-492.

Hartmann, H.T., Kester, F.T., Davies Jr. and Geneve. R.L. (1997). Instructors Manual for Plant Propagation. **In:** Principles and Practices. Prentice-Hall, Englewood Cliffs, NJ, USA. p. 151.

Hernandez, H.M., Gomez-Hinostrosa, C. and Barcenas, R.T. (2001). Diversity, spatial arrangement, and endemism of *Cactaceae* in the Huizache area, a hot-spot in the Chihuahuan Desert. *Int. Biodivers. & Conserv*. **10**(7): 1097-1112.

Hunter, M. (1985). In Defense of *Opuntia. Cact. Succ. J.* **57**: 94-96.

Infante, R. (1992). *In vitro* axillary shoot proliferation and somatic embryogenesis of yellow pitaya *Mediocactus coccineus*. *Plant Cell, Tissue and Organ Cult.* **31**: 155-159.

Inglese, P., Barbera, G. and La Mantia, T. (1995). Research strategies and improvement of Cactus pear *Opuntia ficus indica*, fruit quality and production. *J. Arid Environ*. **29**: 455-468.

Jagatheeswari, D. and Ranganathan, P. (2012). Studies on Micropropagation of *Stevia rebaudiana*. *Int. J. of Pharml. & Biol*. **3**(2): 315-320.

Johnson, J.L. and Emino, E.R. (1979). Tissue culture propagation in the *cactaceae*. *J. Cact. Succ. Soc. Amer*. **51**:275–277.

Joseph, B., Dubrovsky, B., Gretchen, B., North, B. and Nobel, P.S. (1998). Root growth, developmental changes in the apex, and hydraulic conductivity for *Opuntia ficus indica* during drought. *New Phytol*. **138**: 75-82.

Joshi, M. and Dhar, U. (2003). *In vitro* propagation of *Saussurea obvallata* (DC.) Edgew. - an endangered ethnoreligious medicinal herb of Himalaya. *Plant Cell Rep.* **21**(10): 933-939.

Juarez, M.C. and Passera, C.B. (2002). *In vitro* propagation of *Opuntia ellisiana* and acclimatization to field conditions. *Biocell*. **26**: 319-324.

Kelly, J. (2009). How to Propagate Agaves and Cacti from Cuttings and Seed. *Afr. J. Agric. Res*. **11**(10): 898-900.

Khalafalla, M.M., Abdellatef, E., Ahmed, M.M. and Osman M.G. (2007). Micropropagation of cactus (*Opuntia ficus indica*) as strategic tool to combat desertification in arid and semi arid regions. *Int. J. Sustain. Crop Prod.* **2**(4): 1-8.

Kuti, J. O. (2004). Antioxidant compounds from four *Opuntia* Cactus pear fruit varieties. *Food chem.* **85**(4): 527-533.

Landsmann, J., Llamoca-Zarate, R.M., Aguiar, L.F. and Campos, F.A.P. (1999). Whole plant regeneration from the shoot apical meristem of *Opuntia ficus indica*. *J. Appl. Bot.* **73**: 83–85.

Lazcano, C.A., Davies, T.F., Estrada-Luna, A.A. and Durray, S.A., (1999). Propagation of Prickly pear *Opuntia amyclaea*. *Afr. J. Agric. Res*. **10**: 355-357.

Lema-Ruminska, J. and Kulus, D. (2014). Micropropagation of Cacti - a review. *Haseltonia*. **19**: 46-63.

Lema-Ruminska, J. and Kulus, D. (2012). Induction of somatic embryogenesis in *Astrophytum asterias* in the aspect of light conditions, auxin and 2,4-D concentrations. *Acta Scientiarum Polonorum, Hortorum Cultus*. **11**(4): 77-87.

Lizalde, V.H.J., Perez-Molphe-Balch, E., Davila, F.C.A. and Perez, R.M.E. (2003). Propagation of nine Mexican Cacti species by tissue culture techniques. *Scientiae Naturae*. **5**: 21-31.

Machado, M.D.F.P.S. and Prioli, J. (1996). Micropropagation of *Cereus peruvianus mill* by areole activation. *In Vitro Cell Dev. Biol. Plant.* **32**: 199-203.

Maina, S.M., Quinata, E., Kiran, K., Sharma, K., Simon, T., Moses, G. and Santie, M.D.V. (2010). Surface sterilant effect on the regeneration efficiency from cotyledon explant varieties adapted to eastern and Southern Africa. *J. Biotech*. **9**(20): 2866- 2871.

Malda, G., Backhaus, R.A. and Martin, C. (1999). Alterations in growth and crassulacean acid metabolism (CAM) activity of *in vitro* cultured cactus. *Plant Cell Tiss. Org. Cult*. **58**: 1-9.

Maran, J.P., Manikandan, S. and Mekala, V. (2013). Modeling and optimization of betalain extraction from *Opuntia ficus indica* using Box–Behnken design with desirability function. *Ind. Crops Prod.* **49**: 304-311.

Mauseth. J.D. (1979). A new method for the propagation of Cacti, sterile culture of axillary buds. *J. Cact. Succ. Soc. Amer.* **51**: 186-187.

Mayes, V., Lacy, B.B., Ahasteen, J., and Chee, J. (2012). Nanise': A Navajo herbal: One Hundred Plants from the Navajo Reservation. **In:** Chandler, AZ. N. Lorraine, (ed.). Five Star Publications. Arizona, USA. p. 43.

Midey, C. (2005). A magical plant. **In:** Cactus. J. Deutsch and N. Murakhver, (ed.). The Arizona Republic. Arizona, USA. p. 33.

Mohamed-Yasseen, Y., Barringer, S.A., Splittstoesser, W.E. and Schnell. J. (1995). Rapid propagation of tuna (*Opuntia ficus indica*) and plant establishment in soil. *Plant Cell, Tissue and Organ Cult*. **42**:117–119.

Mulas, M., Spano, D., Pellizaro, G. and Hallewin, G. (1992). Rooting of *Opuntia ficus indica*, young cladodes. *Adv. Hortic. Sci*. **6**:44-46.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum***. 15**(3): 473-497.

Myeong, I.J., Chang-Hui, C. and Jung-Myung, L. (2004). Production and breeding of Cacti for grafting in Korea. *Hortic. Sci*. **44**: 7-10.

Nobel, J.L. (1995). Changes in water potential and its component during shoot formation in Cacti callus. *J. Plant Physiol*. **45**: 92-97.

Ogburn, R.M. and Edwards, E.J. (2010). The ecological water use strategies of succulent plants. *Adv. Bot. Res.* **55**: 179-225.

Papafotiou, M., Balotis, G. N., Louka, P. T. and Chronopoulos, J. (2001). *In vitro* plant regeneration of *Mammillaria elongata* normal and cristate forms. *Plant Cell, Tissue and Organ Cult*. **65**: 163-167.

Perez-Molphe-Balch, E., Perez Reyes, M. E., Villalobos Amador, E., Meza Rangel, E., Morones Ruiz, L. R. and Lizalde Viramontes, H. J. (1998). Micropropagation of 21 species of Mexican Cacti by axillary proliferation. *In Vitro Cell. Dev. Biol. Plant.* **34**: 131-135.

Perez-Molphe-Balch, E., Perez-Reyes, M. E., Davila-Figueroa, C. A. and Villalobos-Amador, E. (2002). *In vitro* propagation of three species of columnar Cacti from the Sonoran Desert. *Hort. Sci.* **37**: 693-696.

Perez-Molphe-Balch, E., Perez-Reyes, E., Villalobos-Amador, E., Meza-Rangel, L. R., Morones R. and Lizalde-Viramontes, H. (1998). Micropropagation of 21 species of Mexican Cacti by axillary proliferation. *In Vitro Cell. Dev. Biol. Plant.* **34**: 131–135.

Phillips, G.C. and Collins G.B. (1979). *In vitro* tissue culture of selected legumes and plant regeneration of red clover. *Crop Sci.* **19**: 59-64.

Poljuha, D., Balen, B., Bauer, A., Ljubesic, N. and Krsnik, M. (2003). Morphology and ultrastructure of *Mammillaria gracillis* in *in vitro* culture. *Plant Cell, Tissue and Organ Cult*. **75**: 117-123.

Quiala, E., Matos, J., Montalvo, G., Feria, M., Chavez, M., Capote, A., Perez, N., Barbon, R. and Kowalski, B., (2009). *In vitro* propagation of *Pilosocereus robinii*, an endemic and endangered cactus. *J. Prof. Assoc. Cactus Dev.* **11**: 18-25.

Rajam, S.P.N. (2006). The role of endogenous auxin in root initiation. *Plant Growth Regul.* **13**(1): 77-84.

Ramirez-Carvajal, G.A., Morse, A.M., Dervinis, C. and Davis, J.M., (2009). The cytokinin type-B response regulator PtRR13 is a negative regulator of adventitious root development in Populus. *Plant Physiol*. **150**(2): 759-771.

Resende G.A., Mangolin, C.A. and Machado, M.D.F. (2010). Somaclonal variation in *Cereus peruvianus* and its potential to generate new varieties and broaden the species's genetic basis. *J. Appl. Genet.* **21**(1): 33-42.

Retes-Pruneda, J.L., Valadez-Aguilar, M.L., Perez-Reyes, M.E. and Perez-Molphe-Balch, E. (2007). *In vitro* propagation of *Echinocereus, Escontria, Mammillaria, Melocactus and Polaskia* species. *Boletin de la Sociedad Botanica de Mexico*. **81**: 7-16.

Rosa-Carrillo, M.L., Dominguez-Rosales, M.S., Perez-Reyes, M.E. and Perez-Molphe-Balch, E. (2012). Culture and *in vitro* propagation of threatened *Turbinicarpus* genus Cacti. *Interciencia.* **37**: 114-120.

Rosas, M., Monroy, R., Moebius G. K. and Chavez A. V. M. (2001). Micropropagation of *Turbinicarpus laui* , an endemic and endangered species. *In Vitro Cell. Dev. Biol. Plant*. **37**: 400-404.

Ruvalcaba-Ruiz, D., Rojas-Bravo, D. and Valencia-Botin, A.J. (2010). *In vitro* propagation of *Coryphantha retusa* an endemic and endangered cactus. *Trop. Subtrop. Agroecosystems*. **12**: 139-143.

Schenk, R.U. and Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bet.* **50**: 199-204.

Serrano, C.R. and Silva, J.A.T.D. (2008). Ornamental and Plant Biotechnology. **In:** Gene trapping and its applications in seed floricultural biotechnology. J. A. Silva, (ed.). Global Science Books, Abasolo, Mexico. pp. 119-226.

Shedbalkar, U.U., Adki, V.S., Jadhav, J.P. and Bapat, V.A. (2010). *Opuntia* and other Cacti: applications and biotechnological insights. *Trop. Plant Biol.* **3**(3):136-150.

Soltero, Q.R. (1996). Micropropagation of two species of *Strombocacti.* Ph.D. thesis, CUCEI, Guadalajara, Mexico.

Sriskandarajah and Serek C. (2004). Regeneration from Phylloclade Explants and Callus Cultures of *Schlumbergera* and *Rhipsalidopsis*. *Plant Cell, Tissue and Organ Cult.* **78**(1): 75-81.

Srivastava, N., Barkha, K., Vikas, S., Yogesh, K.N., Dobriyal, A.K., Sanjay, G. and Vikash, S.J. (2010). Standardization of sterilization protocol for micropropagation of *Aconitum heterophyllum* - An endangered medicinal herb. *Acad. Arena*. **2**(6): 37-42.

Synman, H.A. (2006). A greenhouse study on root dynamics of Cactus pears, *Opuntia ficus indica* and *O. robusta. J. Arid Environ*. **65**: 529-542.

Taylor, N.P., Bonatelli I.A.S., Zappi D.C. and Moraes E.M. (2013). Usefulness of cpDNA markers for phylogenetic and phylogeographic analyses of closely related Cactus species. *Genet. Molec. Res.* **12**: 4579-4585.

Valdez, C.A.F. and Osorio, G.A. (1997). *Opuntia* based ruminant feeding systems in Mexico. *J. Prof. Assoc. Cactus Dev*. **2**: 3-8.

Vinas, M., Fernandez-Brenes, M., Azofeifa, A. and Jimenez, V.M. (2012). *In vitro* propagation of purple pitahaya. *In Vitro Cell. Dev. Biol. Plant.* **48**(5): 469-477.

Vyskot, B. and Jara, Z. (1984). Clonal propagation of Cacti through axillary buds in *in vitro. J. Hort. Sci.* **59**:449–452.

Wakhlu, A. K., and Bhau, B. S. (2000). Callus formation and plant regeneration from tubercles of *Coryphantha elephantidens*. *In Vitro Cell. Dev. Biol. Plant.* **36**(3): 211-214.

APPENDICES

APPENDICES

Appendix I. Composition of Duchefa Biochemic MS (Murashige and skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic Acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L Manufacturing Company: Duchefa Biochem

Appendix II: Effect of different concentration of BA on shoot induction potentiality in Cactus

BA (mg/L)	Shoot induction potentiality	
	Percent of explant showing shoot induction	**Days to shoot induction**
0.0	32.62	20.6 a
1.0	43.72	17.6 b
1.5	55.96	13.4 c
2.0	66.44	10.8 d
2.5	93.64	5.4 e
3.0	69.76	10.2 d
CV(%)	-	4.55
LSD(0.05)	-	0.7722

Appendix III: Effect of different concentration of IBA on root induction potentiality at different WAI

IBA (mg/L)	Root induction potentiality				
	Percent of explant showing root induction	Days to root induction	Number of root/explant		
			2 WAI	3 WAI	4 WAI
0.0	38.9	20.6 a	0.2 d	1.0 d	1.4 d
0.5	46.3	16.6 b	0.2 d	1.4 cd	1.8 d
1.0	57.9	13.6 c	1.2 c	1.8 c	2.6 c
1.5	70.1	11.6 d	1.8 b	2.8 b	3.6 b
2.0	93.8	5.4 f	4.8 a	6.4 a	6.8 a
2.5	70.8	10.6 e	2.2 b	3.0 b	3.6 b
CV(%)	-	4.19	14.01	8.29	5.65
LSD(0.05)	-	0.7150	0.5329	0.6537	0.6741

Appendix IV. Analysis of variance on days to shoot induction with BA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	747.600	149.520	427.20	0.0000
Error	24	8.400	0.350	-	-
Total	29	756.000	-	-	-
CV(%)	4.55	-	-	-	-
LSD Value	0.7722	-	-	-	-

Appendix V. Analysis of variance on length of shoot with BA in 2 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	23.5720	4.71440	1430.77	0.0000
Error	24	0.0791	0.00330	-	-
Total	29	23.6511	-	-	-
CV(%)	7.97	-	-	-	-
LSD Value	0.0749	-	-	-	-

Appendix VI. Analysis of variance on length of shoot with BA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	87.5385	17.5077	4020.14	0.0000
Error	24	00.1045	0.0044	-	-
Total	29	87.6430	-	-	-
CV(%)	4.49	-	-	-	-
LSD Value	0.0861	-	-	-	-

Appendix VII. Analysis of variance on length of shoot with BA in 4 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	170.283	34.0565	5128.99	0.0000
Error	24	0.159	0.0066	-	-
Total	29	170.442	-	-	-
CV(%)	3.78	-	-	-	-
LSD Value	0.1064	-	-	-	-

Appendix VIII. Analysis of variance on number of shoot with BA in 2 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	66.0000	13.2000	44.00	0.0000
Error	24	7.2000	0.3000	-	-
Total	29	73.2000	-	-	-
CV(%)	5.09	-	-	-	-
LSD Value	0.7150	-	-	-	-

Appendix IX. Analysis of variance on number of shoot with BA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	63.1000	12.6200	47.33	0.0000
Error	24	6.4000	0.2667	-	-
Total	29	69.5000	-	-	-
CV(%)	5.7	-	-	-	-
LSD Value	0.6741	-	-	-	-

Appendix X. Analysis of variance on number of shoot with BA in 4 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	97.200	19.4400	83.31	0.0000
Error	24	5.600	0.2333	-	-
Total	29	102.800	-	-	-
CV(%)	4.19	-	-	-	-
LSD Value	0.6305	-	-	-	-

Appendix XI. Analysis of variance on days to root with IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	682.667	136.533	455.11	0.0000
Error	24	7.200	0.300	-	-
Total	29	689.867	-	-	-
CV(%)	4.19	-	-	-	-
LSD Value	0.7150	-	-	-	-

Appendix XII. Analysis of variance on number of root with IBA in 2 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	76.3000	15.2600	91.56	0.0000
Error	24	4.0000	0.1667	-	-
Total	29	80.3000	-	-	-
CV(%)	14.01	-	-	-	-
LSD Value	0.5329	-	-	-	-

Appendix XIII. Analysis of variance on number of root with IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	95.867	19.1733	76.69	0.0000
Error	24	6.000	0.2500	-	-
Total	29	101.867	-	-	-
CV(%)	8.29	-	-	-	-
LSD Value	0.6527	-	-	-	-

Appendix XIV. Analysis of variance on number of root with IBA in 4 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	93.900	18.7800	70.43	0.0000
Error	24	6.400	0.2667	-	-
Total	29	100.300	-	-	-
CV(%)	5.65	-	-	-	-
LSD Value	0.6741	-	-	-	-

Appendix XV. Analysis of variance on length of root with IBA in 2 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	37.1350	7.42700	655.32	0.0000
Error	24	0.2720	0.01133	-	-
Total	29	37.4070	-	-	-
CV(%)	10.04	-	-	-	-
LSD Value	0.1390	-	-	-	-

Appendix XVI. Analysis of variance on length of root with IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	96.7108	19.3422	1079.76	0.0000
Error	24	0.4299	0.0179	-	-
Total	29	97.1407	-	-	-
CV(%)	7.99	-	-	-	-
LSD Value	0.1747	-	-	-	-

Appendix XVII. Analysis of variance on length of root with IBA in 4 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	5	167.677	33.5354	1983.95	0.0000
Error	24	0.406	0.0169	-	-
Total	29	168.082	-	-	-
CV(%)	5.81	-	-	-	-
LSD Value	0.1697	-	-	-	-

Appendix XVIII. Analysis of variance on days to Shoot induction with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	1455.60	60.6500	134.18	0.0000
Error	100	45.20	0.4520	-	-
Total	124	1500.80	-	-	-
CV(%)	5.98	-	-	-	-
LSD Value	0.8436	-	-	-	-

Appendix XIX. Analysis of variance on number of Shoot induction in 2 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	158.000	6.58333	31.65	0.0000
Error	100	20.800	0.20800	-	-
Total	124	178.800	-	-	-
CV(%)	12.81	-	-	-	-
LSD Value	0.5723	-	-	-	-

Appendix XX. Analysis of variance on number of Shoot induction in 3 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	164.032	6.83467	25.13	0.0000
Error	100	27.200	0.27200	-	-
Total	124	191.232	-	-	-
CV(%)	7.60	-	-	-	-
LSD Value	0.6544	-	-	-	-

Appendix XXI. Analysis of variance on number of Shoot induction in 4 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	277.312	11.5547	38.52	0.0000
Error	100	30.000	0.3000	-	-
Total	124	307.312	-	-	-
CV(%)	4.86	-	-	-	-
LSD Value	0.6873	-	-	-	-

Appendix XXII. Analysis of variance on length of Shoot induction in 2 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	64.3741	2.68225	1789.12	0.0000
Error	100	0.1499	0.00150	-	-
Total	124	64.5240	-	-	-
CV(%)	5.07	-	-	-	-
LSD Value	0.0486	-	-	-	-

Appendix XXIII. Analysis of variance on length of Shoot induction in 3 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	249.190	10.3829	3074.42	0.0000
Error	100	0.338	0.0034	-	-
Total	124	249.528	-	-	-
CV(%)	3.77	-	-	-	-
LSD Value	0.0729	-	-	-	-

Appendix XXIV. Analysis of variance on length of Shoot induction in 4 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	601.736	25.0723	11152.19	0.0000
Error	100	0.225	0.0022	-	-
Total	124	601.961	-	-	-
CV(%)	2.00	-	-	-	-
LSD Value	0.0595	-	-	-	-

Appendix XXV. Analysis of variance on days to root induction with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	1262.43	52.6013	113.36	0.0000
Error	100	46.40	0.4640	-	-
Total	124	1308.83	-	-	-
CV(%)	5.83	-	-	-	-
LSD Value	0.8547	-	-	-	-

Appendix XXVI. Analysis of variance on number of root induction in 2 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	171.328	7.13867	41.50	0.0000
Error	100	17.200	0.17200	-	-
Total	124	188.528	-	-	-
CV(%)	6.32	-	-	-	-
LSD Value	0.5204	-	-	-	-

Appendix XXVII. Analysis of variance on number of root induction in 3 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	193.472	8.06133	21.67	0.0000
Error	100	37.200	0.37200	-	-
Total	124	230.672	-	-	-
CV(%)	5.08	-	-	-	-
LSD Value	0.7653	-	-	-	-

Appendix XXVIII. Analysis of variance on number of root induction in 4 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	207.200	8.63333	26.98	0.0000
Error	100	32.000	0.32000	-	-
Total	124	239.200	-	-	-
CV(%)	4.64	-	-	-	-
LSD Value	0.7098	-	-	-	-

Appendix XXIX. Analysis of variance on length of root induction in 2 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	85.9376	3.58073	273.25	0.0000
Error	100	1.3104	0.01310	-	-
Total	124	87.2481	-	-	-
CV(%)	7.20	-	-	-	-
LSD Value	0.1436	-	-	-	-

Appendix XXX. Analysis of variance on length of root induction in 3 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	277.316	11.5548	952.02	0.0000
Error	100	1.214	0.0121	-	-
Total	124	278.530	-	-	-
CV(%)	6.09	-	-	-	-
LSD Value	0.1382	-	-	-	-

Appendix XXXI. Analysis of variance on length of root induction in 4 WAI with BA+IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F - Value	Probability
Treatment	24	484.526	20.1886	2197.47	0.0000
Error	100	0.919	0.0092	-	-
Total	124	485.445	-	-	-
CV(%)	3.97	-	-	-	-
LSD Value	0.1203	-	-	-	-