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Effect of plant growth regulators on regeneration of the endangered medicinal plant *Calligonum comosum* L. Henry in the Kingdom of Bahrain

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The use of *in vitro* techniques for conserving plant biodiversity and protecting rare and endangered multipurpose plant species is considered as one of the most important *ex-situ* conservation policies. Development of an efficient *in vitro* regeneration protocol of *Calligonum comosum* is important and that has achieved to protect the endangered multipurpose medicinally important desert plant in the Kingdom of Bahrain. Nodal segments were used as explants source and the effect of various plant growth regulators (PGRs) were studied for responses and to regenerate the whole plants in modified Murashige and Skoog (MS) media through direct and indirect organogenesis via callus induction. 50% explants of *C. comosum* responded to initiate shoot in presence of 4.44 µM BAP with 2.68 µM NAA after four weeks of culture while 40% shoot initiation response was the highest value in presence of 9.29 µM KI with 5.37 µM NAA after 4 weeks of culture among the treatments of KI with NAA. The highest callus induction rate of 100% was found in media containing 9.29 µM KI and 5.37 µM NAA after four weeks. Multiple initial shoots those originated from nodal segments develop calli and showed organogenic differentiation of shoots in presence of BAP and IAA. The highest shoot multiplication frequency of 15 was observed while the shoots initiated in media contained 4.44 µM BAP with 2.85 µM IAA and were transferred to 8.56 µM IAA with 2.22 µM BAP. Shoot multiplication and shoot regeneration capacity was compared in different media and the highest performance of 234 shoots /explants after second multiplication was observed while shoots initiated in presence of 13.3 µM BAP and 5.71 µM IAA. As a precautionary approach to conserve the endangered medicinal plant species in the Kingdom of Bahrain, the application of *in vitro* culture is considered as an important alternative method in *ex situ* conservation strategy in the present study.

Key words: *Calligonum comosum,* endangered plants, *ex situ* conservation, *in situ* conservation, organogenesis, plant regeneration, tissue culture.

INTRODUCTION

The Kingdom of Bahrain is an aggregate of a group of scattered islands forming an archipelago lying almost in the middle of the Arabian Gulf. The temperature is generally high with an average of 35°C, ranging from 14 to 41°C. The average rainfall is 74 mm/year, varying from 39 to 128 mm/ year. Bahrain was relatively rich in

biodiversity with 323 species of flora, those were important in relation to environment protection and national interest (El-Oqlah and Abbas, 1994). Among them, 25% of plants are used as medicinal herb by Bahrain and also by others in the Arabian Peninsula or neighboring countries (Jameel et al., 2010). The unprecedented socio-economic development in the Kingdom of Bahrain has caused part of habitat destruction, widespread ecological modification and biodiversity degradation. Hence, many plant species has became threatened and some have been considered as endangered in Bahrain (Al-Eisawi, 2003). Preserving global biodiversity is a priority project in strategic conservation plan that was designed to engage public policy and concerns affecting local, regional and global scales of communities, ecosystems and cultures (Gascon et al., 2007). *In situ* and *ex situ* conservation is applied according to the need of preservation process. *In situ* conservation is the most valuable and effective method, relies upon the conservation of plants in their living forms, particularly in their natural conditions and habitats. Considering the importance of conserving biodiversity, Al-Areen Wildlife Reserve in the Kingdom of Bahrain established in 1976 (Khanna, 2012). Under certain specific conditions, *ex situ* methods in conservation of some endangered plant species using plant tissue culture has gained a lot of interest in different aspect (Paunescu, 2006). The use of *in-vitro* techniques increase the chances of recovery of endangered species and has reduced the risk of extinction (Nadeem et al., 2000; Anburaj et al., 2011). Similarly, *in vitro* culture commonly known as tissue culture (TC) not only offer the possibilities of faster multiplication of clones of endangered plant species for conservation of genotypes, but also help to preserve some specific cells, tissues, organs in a special way for future use (Benson, 2003; Khan et al., 2012). The stimulation of endogenous growth substances by the addition of exogenous growth regulators to the nutrient media promote cell division, cell growth and differentiation of plant organs (Aboel-nil, 1997). The induction of somatic embryogenesis and organogenesis help to develop large number of regenerated plants within a short period of time by the differentiation of different types of tissues (Tripathi and Tripathi, 2003). *In vitro* plant regeneration via organogenic response of explants either direct or indirect way through axillary and / adventitious shoot regeneration is a well recognized method in obtaining large number of plants (Li et al., 2002; Fatima et al., 2009; Saini et al., 2011).

Calligonum comosum L. Henry, belongs to the family of Polygonaceae, under the local name “arta” (Muschler, 1912) and is considered as one of the endangered multipurpose desert medicinal plants in the Kingdom of Bahrain. It is perennial, small leafless woody shrub, strictly psammophil, grows in arid, sandy ecosystem which may attain a height of 2 to 3 m. Flowers are small, petals are white in color with red anthers during early spring (March and April) and young fruits are red spiny (soft) capsules (Karim, 2003). The plant is a rich source of several secondary metabolites such as flavonoids, alkaloids and phenols. It is traditionally used in folk medicine to treat rural population from microbial infections. Anthraquinones and terpenoids of *Calligonum* showed high antimicrobial potentiality (Zaki et al., 1984), anti-listerial activity (Riadh et al., 2011), anti-inflammatory as well as antiulcer activity (Liu et al., 2001). Rapid urbanization and industrialization, uncontrolled exploittation factors are correlated with loss of natural genetic resources in Bahrain (Al-Eisawi, 2003). The application of tissue culture as a biotechnological tool for the production of large number of plants within short time period particularly in the conservation of threatened medicinally important multipurpose plants has gained huge interest in the last two decades. In spite of extensive advertisement in world-wide research interests on *in vitro* multiplication method to conserve biodiversity as well as to preserve endangered medicinally important plants and pharmaceutically important compounds, no strategic ways have been used in Bahrain. Considering the above points, the objective of the present study was to investigate the role of plant growth regulators (PGRs) in developing and optimizing the protocol of regeneration of endangered medicinal plant *C. comosum*. It is the first approach in Bahrain of using *in vitro* method to propagate endangered multipurpose desert plants.

MATERIALS AND METHODS

Collection of plant materials

Stem segments of *C. comosum* L. Henry (Figure 1A) were collected from plants growing in the southern part of protected reserve forest area in Al-Areen Wild Life Park of Kingdom of Bahrain (Figure 1B). Stem segments (20 to 30 cm) were collected during March to April in 2012 for tissue culture purpose with special permission from the Park authority. Collected stem parts were cut into pieces, stored at 4ºC in closed plastic bags and were used as source of explants for culture initiation after surface sterilization.

Surface sterilization of explants

The stem segments were surface sterilized following modified

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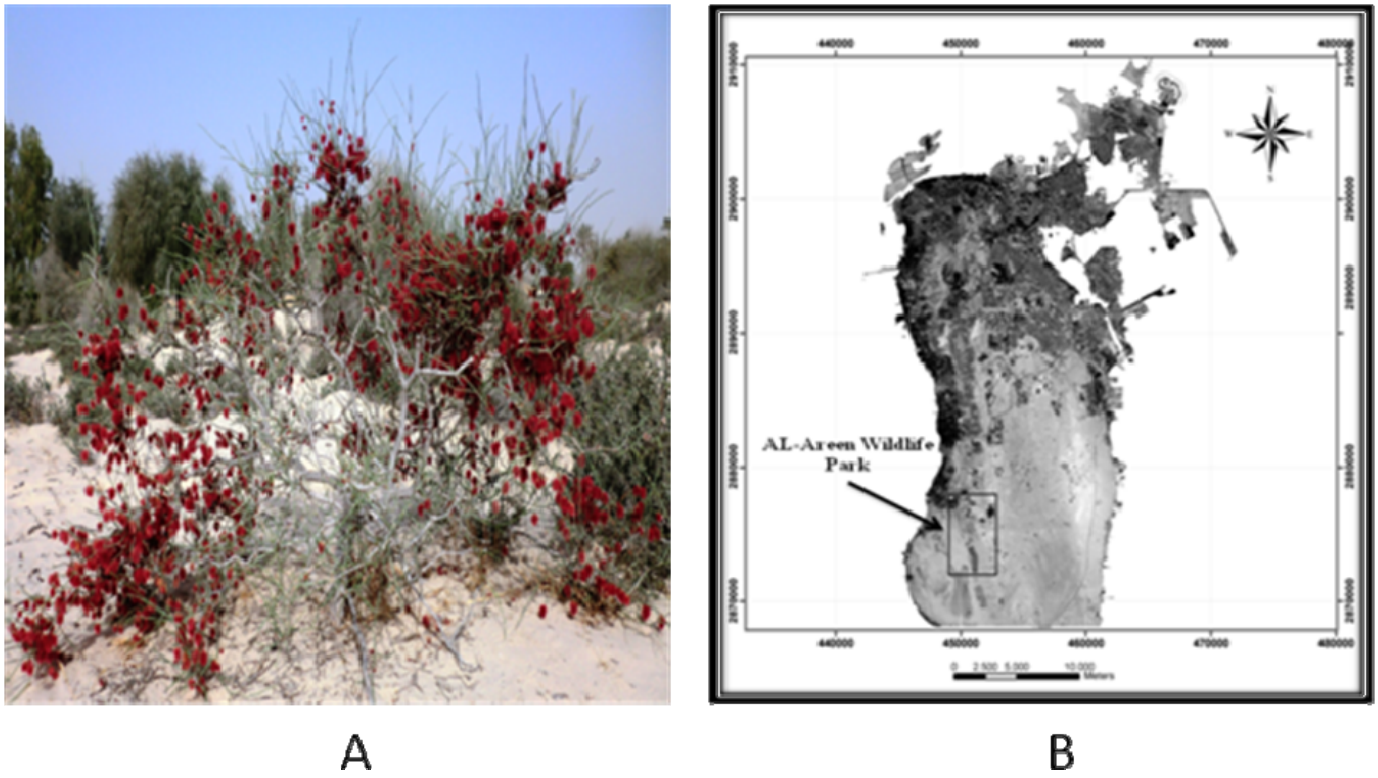

Figure 1. **A.** *Calligonum comosum* L. Henry growing in protected reserve forest area in Al-Areen Wild Life Park of Kingdom of Bahrain. **B.** Al-Areen Wildlife Park (Earth, Google, 2013).

method of Pathak and Hamzah (2008), in running tap water for 5 min to remove dust particles. Then the stem segments were kept for 5 min successively in 1% of lux solution, 0.5% of mercuric chloride solution with few drops of tween 20, 0.1% of copral solution, while the respective solutions were discarded in each step by thorough washing (5 to 6 times) with autoclaved water. Later on, 50% (v/v) Clorox solution (containing 2.625% hypochlorite) was used for 15 min and washed (5 to 6 times) with autoclaved Millipore water to remove hypochlorite nicely. Final washing in 70% ethanol for 30 s was performed before transfer in autoclaved Millipore water until use. The stem segments were sliced into smaller pieces (1.5 to 2 cm), each containing one node used as explants for culture initiation.

Culture media and conditions

The media used throughout culture were modified Murashige and Skoog (MS) media (1962) containing 0.3% of casein hydrolysate, 3% of sucrose, 1 mg/L Nicotinic acid, 1 mg/L Pyridoxine HCl, and 10 mg/L Thiamine HCl. The pH of the medium was adjusted to 5.8. The media were solidified with 0.8 to 0.9% agar and autoclaved at 121°C, for 20 min at 15 psi. Filter (0.22 µm) sterilized plant growth regulators (PGRs) of different combinations and concentrations were added in autoclaved modified MS media and poured in Magenta vessel (30 ml). The cultures were maintained at 16/8 h (light/dark) cycles with cool, white, fluorescent light intensity of 2000 to 2500 LUX, temperature of 24 ± 2 °C, 70 to 80% humidity in culture room.

Treatments for shoot and callus induction, shoot multiplication and rooting

Nodal segments collected from plants (Figure 1A) were used as explants and were cultured *in vitro* in (1) MS media and MS media supplemented (2) 4.44 µM BAP + 2.85 µM IAA; (3) 6.66 µM BAP + 2.85 µM IAA; (4) 8.88 µM BAP + 5.71 µM IAA; (5) 13.32 µM BAP + 5.71 µM IAA; (6) 4.44 µM BAP + 2.68 µM NAA; (7) 6.66 µM BAP + 2.68 µM NAA; (8) 8.88 µM BAP + 5.37 µM NAA; (9) 13.3 µM BAP + 5.37 µM NAA; (10) 4.65 µM KI + 2.85 µM IAA; (11) 6.97 µM KI + 2.85 µM IAA; (12) 9.29 µM KI + 5.71 µM IAA; (13) 13.9 µM KI + 5.71 µM IAA; (14) 4.65 µM KI + 2.68 µM NAA; (15) 6.97 µM KI + 2.68 µM NAA; (16) 9.29 µM KI + 5.37 µM NAA; (17) 13.9 µM KI + 5.37 µM NAA. Based on morphological responses, initially developed shoots in different media were sub-cultured two times for multiplication and regeneration of shoots in modified MS media supplemented with (4) 8.88 µM BAP + 5.71 µM IAA; (12) 9.29 µM KI + 5.71 µM IAA; (22) 8.88 µM BAP + 1.14 µM IAA; (25) 1% charcoal + 4.92 µM IBA; (26) 1% charcoal + 7.4 µM IBA; (27) 1% charcoal + 9.84 µM IBA; (28) 0.93 µM KI + 5.71 µM IAA; (29) 2.32 µM KI + 8.56 µM IAA; (30) 2.85 µM IAA; (32) 0.89 µM BAP + 2.85 µM IAA; (33) 0.89 µM BAP + 5.71 µM IAA; (34) 2.22 µM BAP + 8.56 µM IAA; (35) 2.22 µM BAP + 11.2 µM IAA; (36) 4.44 µM BAP + 2.27 µM TZN + 1.14 µM IAA; (37) 8.88 µM BAP + 4.54 µM TZN; (41) 3% sorbitol + 0.49 µM IBA.

Newly formed microshoots measuring 1-2 cm were cultured in rooting media (1) MS; (4) 8.88 µM BAP + 5.71 µM IAA; (29) 8.56 µM IAA + 2.32 µM KI; (31) 2.85 µM IAA in ½ MS. Rooted plantlets were taken away from culture media, washed nicely in autoclaved

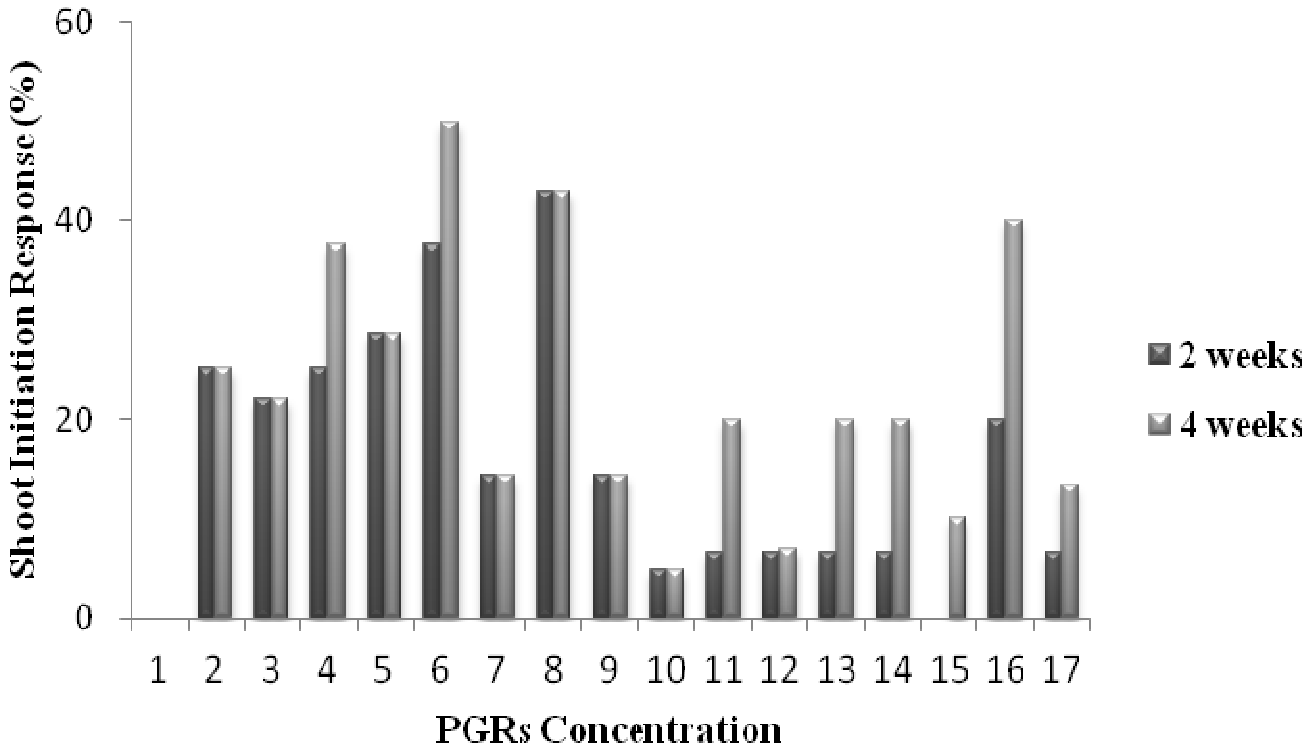

Figure 2. Effect of different plant growth regulators (PGRs) supplemented to modified MS media for shoot initiation response from nodal explants of *C. comosum* after 2 and 4 weeks of culture. Results are percentage of shoot initiation response.

distilled water to remove media from their rooting surface. The plantlets were transplanted in plastic pots containing autoclaved compost soil (1:1 mixture of peat-substrates and potting soil) and were kept in transparent small covered chamber to keep moist. The plants were acclimatized in room conditions at 25±3°C, 16/8 hs photoperiod and watered regularly at 3 days interval. Based on morphological responses of explants, percentage of shoot and callus initiation response, frequencies, percentage of shoot proliferation in first and second transfer, shoot multiplication frequency from developed callus and plant regeneration capacity per explants were calculated. The shoot initiation frequency was measured by calculating the number of shoots initiated/explants. Plant regeneration capacity was calculated by counting mean number of shoots initiated in each step based on shoot initiation frequency of explants multiplied by two cycles of mean proliferation frequencies where shoots initiated direct and indirect way of organogenic response of calli.

Experimental design and statistical analysis

The experiments were carried out by using completely randomized design (CRD) with three replications of each experiment and 3 to 5 explants per replications. Data were analyzed using SPSS (SPSS Inc. Version 17.0), statistical package. Means of 3 replicates per treatment were compared with control as well as multiple treatment groups. Mean comparisons were performed at $P \leq 0.05$ level of significance using one way analysis of variance (ANOVA) according to Duncan's multiple range test (DMRT) using JMP (version 9) statistical software.

RESULTS

Shoot and callus initiation response

Nodal segments (Figure 6A) were cultured on MS media fortified with different concentrations and combinations of cytokinins (BAP and KI) and auxins (IAA and NAA) to study the initial responses. The nodal explants responded by shoot and callus initiation response (Figure 6B and 6C). The percentage of shoot initiation response was scored after two and four weeks of culture (Figure 2). MS medium devoid of PGRs showed no shoot initiation response, explants remained fresh for one to two week then turned necrotic and died. The highest shoot initiation response of 50% was observed in media containing 4.44 µM BAP with 2.68 µM NAA after 4 weeks of culture among the all treatments. While 40% shoot initiation response was the highest value in presence of 9.29 µM KI with 5.37 µM NAA after 4 weeks of culture among the treatments of KI with NAA. Similarly, the highest shoot initiation frequencies of 37.5 and 25% were noticed in media containing 8.8 µM BAP with 5.71 µM IAA and 6.97 µM KI with 2.85 µM IAA after 4 weeks of culture respectively among the treatments of IAA with BAP and

Table 1. Effect of various growth regulators supplemented to modified MS media on *in vitro* shoot initiation response from nodal explants of *Calligonum comosum* (Arta) after 4 weeks of culture.

PGRs combinations	Mean calli number
4.44 µM BAP + 2.68 µM NAA	2.66^{A}
8.88 µM BAP + 5.37 µM NAA	2.66^{A}
8.88 µM BAP + 5.71 µM IAA	1.66^{AB}
13.3 µM BAP + 5.71 µM IAA	1.66^{AB}
4.44 µM BAP + 2.85 µM IAA	1.33^{BC}
6.66 µM BAP + 2.85 µM IAA	1.33^{BC}
6.66 µM BAP + 2.68 µM NAA	1.33^{BC}
9.29 µM KI + 5.37 µM NAA	1.33^{BC}
13.3 µM BAP + 5.37 µM NAA	1^{CD}
4.65 µM KI + 2.85 µM IAA	1^{CD}
6.97 µM KI + 2.85 µM IAA	1^{CD}
13.9 µM KI + 5.71 µM IAA	1^{CD}
13.9 µM KI + 5.37 µM NAA	1^{CD}
9.29 µM KI + 5.71 µM IAA	0.66^{DE}
4.65 µM KI + 2.68 µM NAA	0.33^{EF}
6.97 µM KI + 2.68 µM NAA	0^{G}
Media without PGRs	0^{G}

Results are means of shoots developed per explant of 3 sets of individual experiments. Means followed by the same letter are not significantly different at P ≤ 0.05

KI (Figure 2). The shoot initiation response of 39% was observed in presence of 4.44 µM BAP with 2.68 µM NAA after two weeks of culture, but after 4 weeks it reached to 50%. The highest response of shoot initiation of 28.6% was observed in media supplemented with 13.3 µM BAP and 5.71 µM IAA after two week of culture initiation, while in 4 weeks old culture it reached to 37.5 (Figure 2) among the treatments of BAP with IAA. Similarly, the shoot initiation response increased with time of culture in different media which is a common trend as observed in all treatments. The effect of KI and NAA were less in compare BAP with NAA in shoot initiation response. The highest shoot initiation frequency was 2.66 in presence of (4.44, 8.88) µM BAP with (2.68, 5.37) µM NAA respectively after 4 weeks of culture. Analysis of data showed interactive effects of different culture media containing different concentrations and combinations of PGRs in inducing initial shoots (mean) were statistically significantly different at P ≤ 0.05 level (Table 1). The induction of adventitious shoot from nodal part and induction of callus were observed in all the PGRs treatment, presented in Figure 6B and 6C. Analysis of variance of shoot initiation response with PGRs showed the prominent effect of BAP with NAA and IAA than KI with NAA and IAA to induce initial axiliary shoots. Figure 3 shows the percentage of callus initiation response in presence of different concentrations and combinations of PGRs as presented in different media after 2 and 4 weeks of culture. The callus was initiated from the cut end of the explants which touched the media. The highest callus induction rate of 40 and 100% was found in media containing 9.29 µM KI and 5.37 µM NAA after 2 and 4 weeks of culture of nodal segments respectively among all the treatments. The callus was compact, white to off white in color. The highest response of callus initiation of 60% was found in media supplemented with KI (6.97, 13.9 µM) and IAA (2.85, 5.71 µM) after 4 weeks of culture among all the treatments of IAA with KI. Under similar condition, the same medium constituents showed only 20% callus initiation after 2 weeks of culture. Among all the treatments of BAP and IAA, the highest callus induction response of 87.5% was observed while media was supplemented with 8.88 µM BAP and 5.71 µM IAA after 2 and 4 weeks. The callus was white in color initially but turned brown with time. The highest callus induction rate of 50% was observed in presence of 4.44 µM BAP and 2.68 µM NAA after 4 weeks, while 42.8% response was found in media containing (8.88, 13.3) µM BAP with 5.37 µM NAA after 2 weeks. The callus was initially compact and white in color, later on became friable and loose. There was no response for callus in media without PGRs after 2 and 4 weeks. The highest callus initiation frequency of 7 ± 0.57 was observed on medium supplemented with 8.88 µM BAP and 5.71 µM IAA after 4 weeks (Table 2). Different treatments of PGRs were compared in callus initiation response with control and multiple treatment groups and their means were compared and analyzed by analysis of variance (ANOVA) using Duncan's multiple range test at (P ≤ 0.05) level and showed significant differences. Interactive effects of culture media on development of mean number of calli per explants were statistically significant (P ≤ 0.05) presented in Table 2.

Shoot multiplication and plant regeneration

Initially, developed shoots from different media were transferred in media containing various combinations and concentrations of PGRs for first time multiplication. The effects of BAP, IAA, NAA, KI, TZN and IBA on first time multiplication of initially developed shoots and the response of shoot associated callus were presented in Table 3. The percentage of proliferated shoot from initially developed shoots, callus, shoot multiplication frequency with associated callus growth were compared in different media. The initially developed shoots were transferred in various media and 100% shoot and callus proliferation were noticed mostly. The highest shoot multiplication frequency of 15 was observed while the shoots initiated in media contained 4.44 µM BAP with 2.85 µM IAA and were transferred to 8.56 µM IAA with 2.22 µM BAP. The lowest shoot multiplication frequency

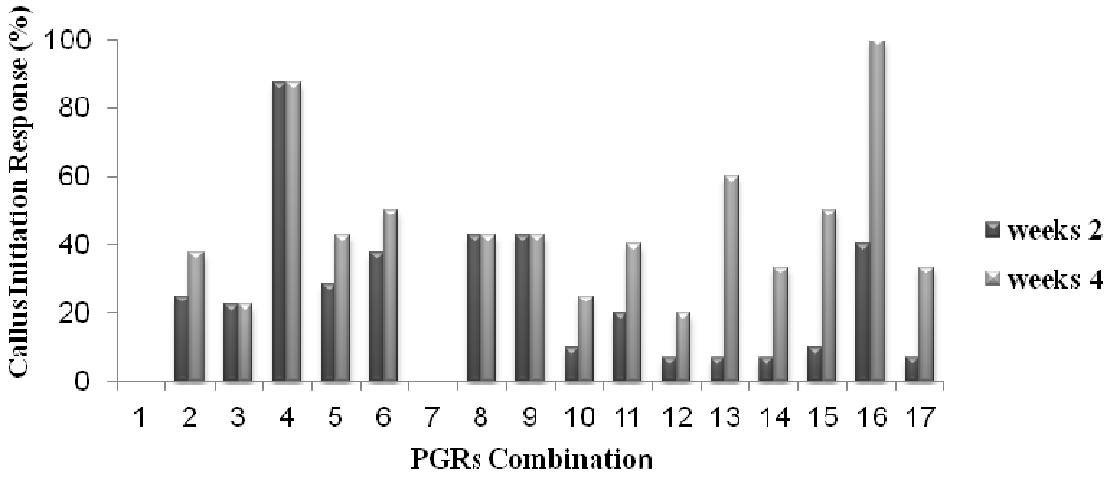

Figure 3. Effect of PGRs supplemented to modified MS media for callus initiation response of *C. comosum* after 2 and 4 weeks of culture. Results are percentage of callus initiation response.

Table 2. Effect of various growth regulators supplemented to modified MS media on *in vitro* callus initiation response from nodal explants of *Calligonum comosum* (Arta) after 4 weeks of culture.

PGRs combinations	Mean calli number
8.88 µM BAP + 5.71 µM IAA	7^{A}
4.44 µM BAP + 2.68 µM NAA	2.66^{B}
4.44 µM BAP + 2.85 µM IAA	2^{BC}
13.3 µM BAP + 5.71 µM IAA	2^{BC}
8.88 µM BAP + 5.37 µM NAA	2^{BC}
6.97 µM KI + 2.85 µM IAA	2^{BC}
6.66 µM BAP + 2.85 µM IAA	1.66^{BCD}
4.65 µM KI + 2.85 µM IAA	1.66^{BCD}
9.29 µM KI + 5.37 µM NAA	1.66^{BCD}
13.3 µM BAP + 5.37 µM NAA	1.33^{CDE}
9.29 µM KI + 5.71 µM IAA	1^{DE}
6.97 µM KI + 2.68 µM NAA	1^{DE}
13.9 µM KI + 5.37 µM NAA	0.66^{EF}
13.9 µM KI + 5.71 µM IAA	0.33^{FG}
4.65 µM KI + 2.68 µM NAA	0.33^{FG}
6.66 µM BAP + 2.68 µM NAA	0^{G}
Media without PGRs	0^{G}

Results are means of calli developed per explant of 3 sets of individual experiments. Means followed by the same letter are not significantly different at P ≤ 0.05.

of 1 was observed while shoot initiated in presence of 13.94 µM KI with 5.71 µM IAA and 9.29 µM KI with 5.71 µM IAA, and were transferred to media containing 11.22 µM IAA with 2.22 µM BAP and 4.44 µM BAP, 2.27 µM TZN with 1.14 µM IAA. Shoot multiplication frequencies after first and second time proliferation were compared and the maximum shoot multiplication was observed in shoot initially developed in media containing 13.32 µM BAP with 5.71 µM IAA (Figure 4). The initially developed shoots in media 2, 4, 5 after transfer to different media (4, 12, 32, 33, 34, 35) developed callus which showed organogenic response efficiently by differentiating a large number of shoots. The callus showed differentiation of shoots were granular, compact, white to green in color (Figure 6D) but became brown to red in color, more compact, nodular and more embryogenic nature in subsequent culture (Figure 6E). The initially developed callus from explants being compact in nature did not differentiated into shoots in different media after (data not shown), but the calli developed after transfer of the newly differentiated shoots, were more compact and nodular, embryogenic nature and finally differentiation into large number of shoots (Figure 6F, G, H). Clustered shoots were separated and sub-cultured in the same media for multiplication. The highest shoot regeneration capacity of 234 shoot/explants were observed while initial shoots developed in media 5, were transferred to media 4 and 41 subsequently for shoot differentiation and multiplication (Figure 5). The callus developed from newly developed shoots in different media showed multiplication of shoots and differentiation of calli into new shoots too (Figure 6F, G, H). The highly defined organogenic response of calli in second multiplication cycle was noticed (Figure 6I, J). Multiplied shoots were isolated and further cultured in different rooting media (Figure 6K, L, M) and regenerated plants (Figure 6N) were transferred to soil (Figure 6O).

DISCUSSION

In the present study, both callus initiation and shoot

Table 3. Effect of various growth regulators on first time multiplication of *C. comosum* shoots those were initiated on modified MS medium supplemented with different concentration and combination of PGRs.

Media	% of proliferated shoot	% of proliferated callus (mean)	Shoot multiplication frequency	Callus growth
2 → 12	50	100	2.5	+ +
2 → 32	50	75	4	+ +
2 → 34	100	100	15	+ +
4 → 32	100	100	10	+ +
4 → 33	75	100	8.12	+ +
4 → 34	66.6	100	5	+
5 → 4	50	100	7.5	+ +
5 → 32	100	100	8.5	+
5 → 35	100	100	12.5	+
7 → 22	100	100	10	+ +
7 → 25	100	100	4	+ +
11 → 22	100	100	10	+ +
11 → 36	100	100	2	+ +
13 → 35	100	100	1	+
13 → 37	100	100	5	+ +
14 → 36	100	100	2	+ +
14 → 37	100	100	2	+
15 → 22	100	100	2	+ +
15 → 26	100	100	10	+
16 → 36	100	100	1	+ +
16 → 26	100	100	3	+
17 → 37	100	100	2	+ +
17 → 27	100	100	3	+ +

Results are percentage of proliferated shoots, calli, shoot multiplication frequency and callus growth data of three sets of individual experiments.

initiation response varied in MS medium supplemented with different PGRs. It was evident from this study that the optimum concentration of BAP with NAA was very effective in shoot initiation. Analysis of variance of shoot initiation response with PGRs showed the prominent effect of BAP with NAA and IAA than KI with NAA and IAA to induce initial axillary shoots. Similar effect of BAP with NAA for shoot induction was reported earlier in Bermuda grass (Jain et al., 2005), *Vigna unguiculata* (Muhammad et al., 2008), *Pteris vittata* (Shukla and Khare, 2012). Tripepi (1997) reported that various concentrations of BAP help to induce larger number of adventitious shoot than other PGRs in micropropagation of several ornamental plants. Similarly, in several studies BAP was more effective for axillary shoot initiation from nodal segments of *Melia azedarach* (Sen et al., 2010) and *Bacapa monneiri* (Gurnani et al., 2012). While Nodal segments of *Melissa officinalis* L. showed *in vitro* plantlet development in presence of different concentration of BAP with IAA (Mohebalipour et al., 2012). He also reported that genotypes and culture media differentially responded in organ differentiation and plant regeneration. PGRs concentrations and combinations particularly BAP with NAA and IAA both play important role in shoot initiation response in different degrees. The comparative effect of different PGRs in stimulating shoot multiplication response showed more effectiveness of BAP with NAA than BAP with IAA and it is the fact that total hormonal response has been gathered from initial culture to subsequent cultures. The ratio of auxin and cytokinin 1: 2-1:3 was more effective in organogenic response and adventitious shoot induction was reported in several studies (Makunga and Staden, 2008; Sen et al., 2009).

The highest response of callus initiation of 60% was found in media supplemented with KI (6.97, 13.9 µM) and IAA (2.85, 5.71 µM) after 4 weeks of culture. The callus developed around the cut surface of explants were sub-cultured in MS media supplemented with different concentration and combinations of BAP, KI, IAA and NAA but there was no organogenic response of calli either root or shoot initiation except its own growth. Initially, white to off-white, light yellow, less compact callus developed. Different treatments of PGRs showed significant differences in callus induction frequencies in different

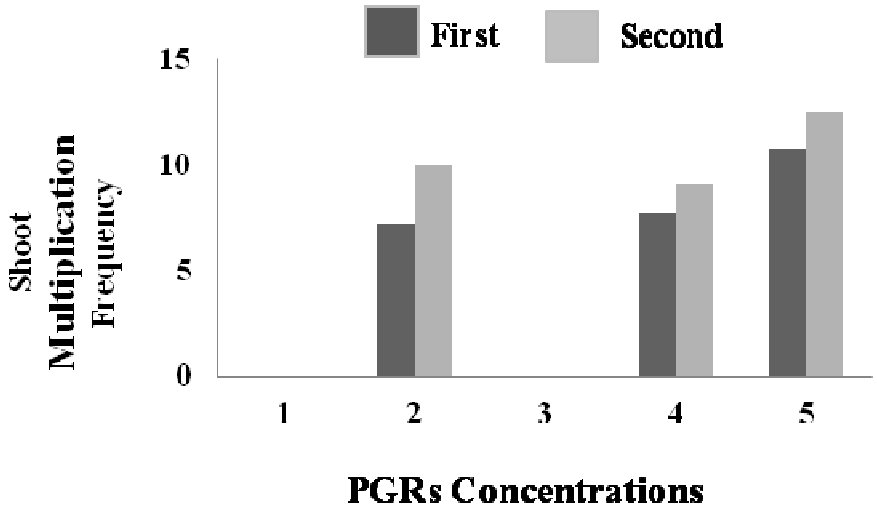

Figure 4. Effect of PGRs on shoot multiplication frequencies of *C. comosum* after 1st and 2nd transfer.

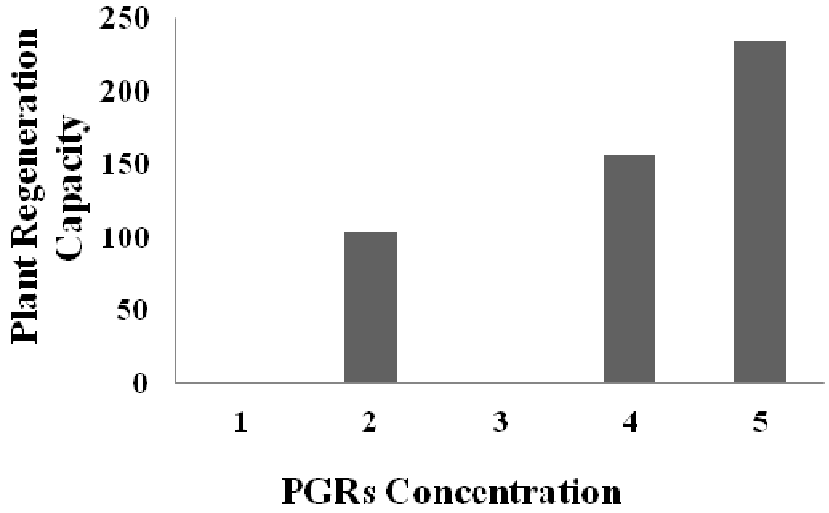

Figure 5. Effect of PGRs in shoot regeneration capacity of *C. comosum* after 2nd transfer.

media but the nature of the initially developed calli were nonembryogenic in all the media. In general, the nature of callus mass was loose, friable and watery in appearance, but with gradual exposure to BAP with IAA in two successive transfers developed compact, hard and more organized calli mass. Moreover, with increased time of culture, calli mass changed its nature, which was explained by Skoog and Miller (1957). They proposed that the type and the concentration of hormone present in medium directly affect the nature, color and biochemical composition of callus. Callus with slow growth rate, friable and watery in appearance has been considered non embryogenic and less regenerative potential (Moris and De-Macan, 1994), this was observed in our experiments also.

Newly differentaiated axillary shoots that developed in presence of 4.4 µM BAP with 2.85 µM IAA, 8.88 µM BAP with 5.71 µM IAA, 13.32 µM BAP with 5.71 µM IAA showed good shoot regeneration capacity during subculture in media containing 8.56 µM IAA with 2.22 µM

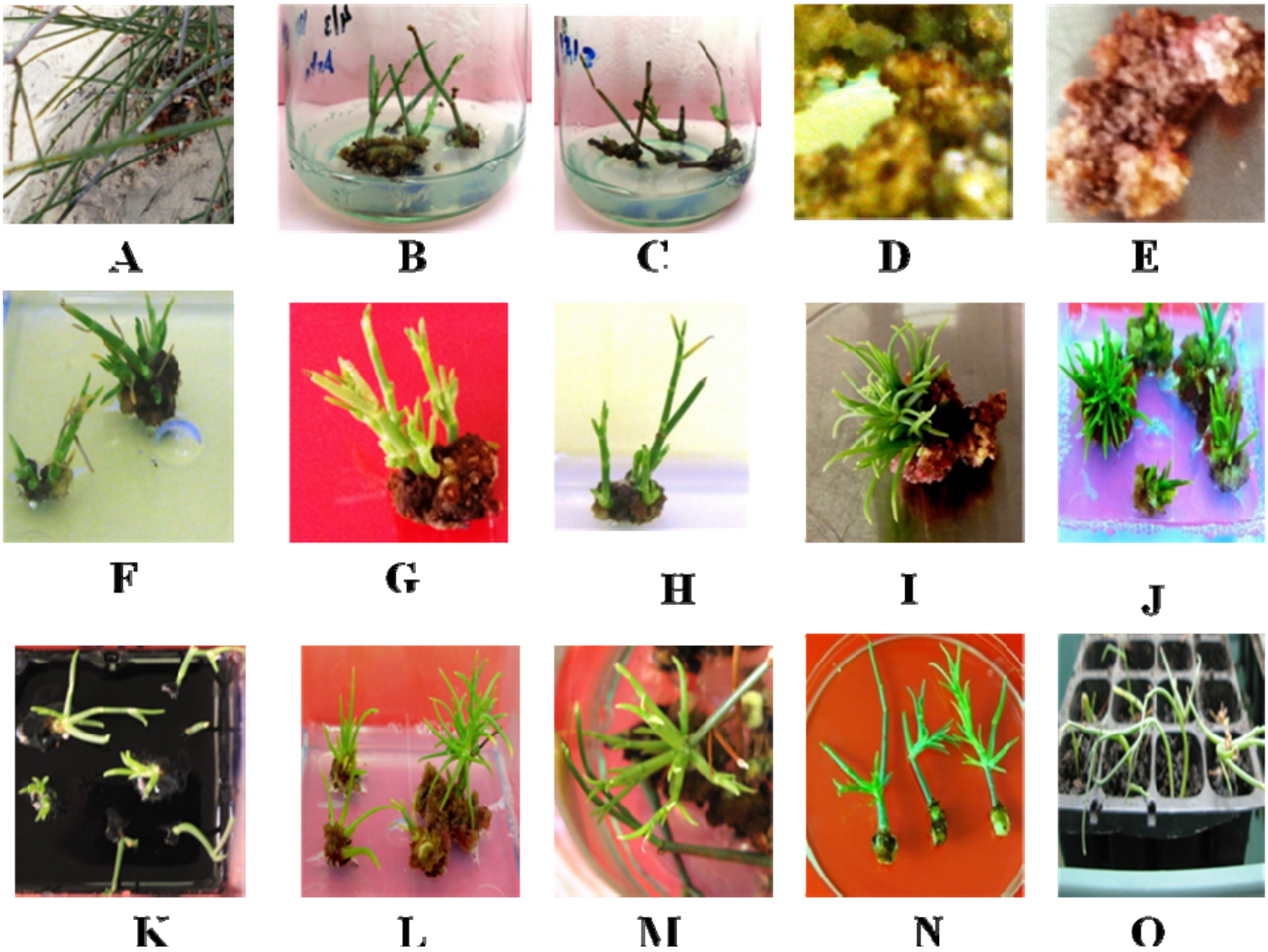

Figure 6. Stages of organogenic response of *C. comosum* in plant regeneration from nodal segments. **A.** Explants were collected from plants growing in Al Areen Wildlife Park in the Kingdom of Bahrain. **B, C.** Stem segments with nodal part in culture media showing shoot and callus initiation response. **D, E.** Callus of *C.comosm* which showed differentiation of shoots. **F, G, H.** Initial shoots showing multiplication of shoots in large number after first transfer in different media. **I, J.** Multiplied shoots were transferred to in different media for proliferation in different media. **K, L, M.** Proliferated shoot were transferred to rooting media. **N.** Micro-shoots showing initial root development. **O.** Plantlets growing in soil pots.

BAP, 2.85 µM IAA with 0.89 µM BAP, 11.22 µM IAA with 2.22 µM BAP, respectively. During this subculture of shoot, calli developed around the new shoot showed differentiation and large number of shoots developed by indirect embryogenesis from calli. The interacting effect of BAP with IAA was more prominent in differentiation of shoot as well as shoot regeneration from calli we observed here. Similar effect of BAP and IAA on shoot regeneration was observed in melon genotypes (Melara and Arias, 2009), and peanut (Iqbal et al., 2011). BAP and IAA are effective to increase the regeneration potential of peanut calli (Iqbal et al., 2011). The highest shoot regeneration capacity of 234 shoots per explant was observed, while shoots were initiated in presence of BAP with IAA, and was subsequently cultured in media containing the same hormones but different concentrations. The synergistic effect of BAP and IAA is more compare to other PGRs in regenerating large number of shoots in this plant. According to Coenen and Lomax (1997), auxins are known to exhibit synergistic, antagonistic and additive interactions with cytokinins at multiple levels depending on the plant species and tissue types in regulating responses. Similarly, Mohebalipour et al. (2012) observed that addition of BAP and IAA to MS medium is more effective than other hormones which significantly increased the number of shoot induction per

explants in most of the genotypes of *M. officinalis* L. The plant regeneration capacity of 2000 to 3000 plants from one shoot after long subculture of *Salvia africana-lutea* L. was reported (Makunga and Staden, 2008) where BA and IAA were used for subcultures. Plant regeneration ability in the culture medium is usually enhanced by the addition of auxins and cytokinins which is very common (Varshney et al., 1996; Fatima et al., 2009). Similarly, the effect of BAP as the most reliable, useful PGR for shoot proliferation, for breaking dormancy in several medicinal plant species were also reported (Tavares et al., 1996; Meszaros et al., 1999; Da Silva et al., 2005; Ghiorghita et al., 2005). The well developed regenerated shoots of *C. comosum* were transferred for rapid multiplication as well as for root development and finally rooted plants were transferred in soil. The current studies highlighted differential response of auxin and cytokinin on direct and indirect shoot regeneration of desert shrub. The success of this protocol offers a high efficient method for micropropagation of *C. comosum* which would be beneficial for the plant tissue culturist, pharmaceutical and nursery industries where regular supply of plants is important part. Moreover, protocol will be helpful in further research in biodiversity conservation of desert plants in this Arab region.

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REFERENCES

Aboel-nil MM (1997). Tissue culture of native plants in the developing countries. Acta Hort. 447: 507-512.

Al-Eisawi D. (2003). Effect of biodiversity conservation on arid ecosystem with a special emphasis on Bahrain. J. Arid Environ. 54:81-90.

Anburaj J, Singh CR, Sundarraj S, Kannan S (2011). *In vitro* regeneration of *Cleome viscose*- an important medicinal herb. J. Cell Mol. Biol. 9(1):37-44.

Benson EE (2003). An introduction to plant conservation biotechnology. In Benson EE (eds.) Plant Conservation Biotechnology. Taylor & Francis Ltd. London, pp. 1-9.

Coenen C, Lomax TL (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci. 2:351-356.

Da Silva S, Sato A, Luiz C, Lage S, Azevedo A, Apparecida EM (2005). Essential oil composition of *Melissa officinalis* L. *in vitro* produced under the influence of growth regulators. J. Braz. Chem. Soc. 16:1387-1390.

El-Oqlah A, Abbas J (1994). A checklist of vascular plants of Bahrain. Dirasat J. 21:95-118.

Fatima Z, Mujib A, Fatima S, Arshi A, Umar S (2009). Callus induction, biomass growth and plant regeneration in Digitalis lanata Ehrh. : influence of plant growth regulators and carbohtdrates. Turk J. Bot. 33:393-405.

Gascon C, Collins JP, Moore RD, Church DR, McKay JE, Mendelson JR (2007). Amphibian Conservation Action Plan. IUCN/SSC Amphibian Specialist Group. Gland, Switzerland and Cambridge, UK, pp. 64-79.

Ghiorghita GI, Mafteli DES, Nicuta DN (2005). Investigations on the *in vitro* morphgenetic reaction of *Melissa officinalis* L. species. Genet. Biol. Mol. 5:119-125.

Gurnani C, Kumar V, Mukhija S, Dhingra A, Rajpurohit S, Narula P (2012). *In vitro* regeneration of Brahmi (*Bacopa monneiri* L. Penn) – A threatened medicinal plant. J. Sci. Eng. Tech. 8:97-99.

Iqbal MM, Nazir F, Iqbal J, Tehrim S, Zafar Y (2011). *In vitro* micropropagation of peanut (*Arachis hypogaea*) through direct somatic embryogenesis and callus culture. Int. J. Agric. Biol. 13:811-814.

Jain M, Chengalrayan K, Gallo-Meagher M, Mislevy P. Mislevy P (2005). Embryogenic callus induction and regeneration in pentaploid hybrid bermudagrass cv. Tifton 89. Crop. Sci. 45:1069-1072.

Jameel A, Al-Laith A, Qaher M (2010). Knowledge, attitudes, and practices regarding medicinal plants used by the indigenous people of Bahrain. Arab Gulf J. Sci. Res. 28:105-117.

Khan S, Al-Qurainy F, Nadeem M (2012). Biotechnological approaches for conservation and improvement of rare and endangered plants of Saudi Arabia. Saudi J. Biol. Sci. 19:1-11.

Khanna A (2012). Implementation of UNCCD in the GCC countries. In: Khanna A, Eds., Global Environmental Governance and Desertification: A Study of Gulf Corporation Council Countries, Concept Publishing Company Pvt. Ltd., New Delhi, India. p.272.

Karim F (2003). *Calligonum comosum* L`Her. In Jongbloed MV Edited - The comprehensive guide to the wild flowers of the United Arab Emirates, Erwda, Emirates Printing Press, Dubai, U.A.E., pp. 56-63.

Li X, Krasnyanski S, Korban SS (2002). Somatic embryogenesis, secondary somatic embryogenesis and shoot organogenesis in Rosa. J. Plant Physiol. 19:313-319.

Liu XM, Zakaria MN, Islam MW, Radhakrishnan R, Ismail A, Chen HB, Chen K and Al-Attas A (2001). Anti inflammatory and anti-ulcer activity of *Calligonum comosum* in rats. Fitoterapia. 72:487-491.

Makunga N, Staden J (2008). An efficient system for the production of clonal plantlets of the medicinally important aromatic plant: *Salvia Africana-lutea* L. Plant Cell, Tiss. Org. Cult. 92:63-72.

Melara MV, Arias AMG (2009). Effect of BAP and IAA on shoot regeneration in cotyledonary explants of Costa Rican melon genotypes. Agron. Costarri. 33:125-131.

Meszaros A, Bellon A, Pinter E, Horvath G (1999). Micropropagation of lemon balm. Plant Cell, Tiss. Org. Cult. 57:149-152.

Mohebalipour N, Aharizad S, Mohammadi S, Motallebiazar A, Arefi H (2012). Effect of plant growth regulators BAP and IAA on micropropagation of Iranian lemon balm (*Melissa officinalis* L.) landraces. J. Food, Agri. Environ. 10(1):280-286.

Moris CF, De Macon VL (1994). Seed Physiology, production and technology. Crop Sci. 34:1324-1329.

Muhammad A, Khalid MK, Sehahattin O (2008). *In vitro* micropropagation from shoot meristems of Turkish cowpea (*Vigna unguiculata* L. cv. Akkiz). Bangladesh J. Bot. 37:149-154.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473-497.

Muschler R (1912). *A Manual Flora of Egypt*. R. Friedlaender & Sohn, Berlin, pp. 257.

Nadeem M, Palni LMS, Purohit AN, Pandey H, Nandi SK (2000). Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. Bio. Cons. 92(1):121-129.

Pathak MR, Hamzah RY (2008). An effective method of sonicxated assisted Agrobacterium-mediated transformation of cheakpeas. Plant Cell, Tiss. Org. Cult. 93:65-71.

Paunescu A (2006). Strategii de conservare a biodiversităţii. In Biodiversitatea de la Concepte Fundamentale la Aplicaţii Biotehnologice, Ed. Acad. Rom., Bucureşti, pp.120-126.

Riadh H, Imen F, Abdelmajid Z , Sinda F (2011). Detection and extraction of anti-listerial compounds from *Calligonum comosum* – medicianl plant from arid regions of Tunisia. Afr. J. Tradit. Complement Altern. Med. 8:322-327.

Saini R, Sharma A, Sharma MM, Batra A (2011). Impact of phytohormones in micropropagation of medicinally potent plant: Boerhavia diffusa L. Int. J. Pharm. Sci. Rev. Res. 8:85-89.

Sen A, Batra A and Rao D (2010). Pivotal Role of Plant Growth Regulators in Clonal Propagation of *Melia azedarach* L. Int. J. Pharm. Sci. Rev. Res. 5:43-49.

Sen A, Sharma MM, Grover D, Batra A (2009). *In vitro* regeneration of *Phyllanthus amarus* Schun. and Thonn. : An important medicinal plant. Our Nat. 7:110-115.

Shukla SP, Khare PB (2012). *In vitro* shoot regeneration via caulogenesis in fern, *Pteris vittata* L. J. Environ. Biol. 33: 683-687.

Skoog F, Miller C (1957). Chemical regulation of growth and organ formation in plant tissue cultures. *In vitro* Sym. Expt. Bio. 11:118-13.

Tavares AC, Pimenta MC, Gonalves MT (1996). Micropropagation of Melissa officinalis L. through proliferation of axillary shoots. Plant Cell Rep. 15:441-444.

Tripathi L, Tripathi JN (2003). Role of biotechnology in medicinal plants. Trop. J. Pharm. Res. 2:243-253.

Tripepi R (1997). Adventitious shoot regeneration. In Geneve R, Preece J, Merkle S (Eds.), Biotechnology of Ornamental Plants. Biotechnology in Agriculture Series. No. 16. CAB International. Wallingford. UK, pp. 45-71.

Varshney V, Kant T, Sharma VK, Rao A, Kothari SL (1996). High frequency plant regeneration from immature embryo cultures of *Triticum aestivum* and *T. durum*. Cereal Res. Commu. 24(4):409-416.

Zaki D, Abd-El-Aziz M, El-Gengeihy S, Morsi N (1984). Antimicrobial potentialities of some Egyptian desert plants. Herba Hungarica. 23:73-84.