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Full Length Research Paper

Establishment of an Efficient *in Vitro* Regeneration Protocol for Mass Propagation via organogenesis of Endangered Plant *Rhanterium epapposum* in Qatar

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Wild plants make an important contribution to the life of local communities. Endangered plant species like *Rhanterium epapposum* olive (Arfaj) were micropropogated through tissue culture technique in order to protect them from extinction and conserving Biodiversity in Qatar. Agriculture Research Department (ARD) has developed an efficient protocol for rapid and large-scale *in vitro* propagation of the native annual plants *Rhanterium epapposum* that can not be propagated on a large-scale by means of seeds and cuttings. Apical bud and nods were collected from wild habitat and cultured on modified Murashige and Skoog (MS) medium supplemented with1.5mg l^{-1} BA (initiation stage) and containing 1mg l^{-1} BAP+ 0.2 mg l^{-1} Kinetin for multiplication stage. A continuous of mass propagation was achieved through three times sub-culture of explants with regular transferring to fresh medium containing the same composition. For rooting stage, elongated shoots were cultured on different strength of Murashige and Skoog media (full and half) supplemented with different auxin. Full MS nutrient medium containing 1 mg l^{-1} IBA increased significantly the number of roots and root length compared with other treatments. The plantlets were adapted in greenhouse conditions successfully. The optimized protocol proposed here can be used to propagate the plants and conserve it from extinction.

Keywords: *In Vitro,* Plant, *Rhanterium epapposum,* organogenesis.

Abbreviations: distilled water: (D.W.); beznzylamino-purine: BAP; sodium dihydrogephosphate: NaH_2PO_4; Kinetin (N^6-furfuryladenine): kin; Indole 3 butyric acid: IBA; Naphthalene acetic acid: NAA; Murashige and Skoog: MS, Agriculture Research Department: ARD

INTRODUCTION

Arfaj is a plant found in Nature as a tree or semi tree characteristics, it has a low woody plant that produces daisy-like flowers and has a cub-shaped root that makes it easy to drag from the ground. The leaves are small and narrow and in late spring, it is covered with straw-yellow flowers about 1cm wide. It flowers from April to May and produces numerous fruits, which forms in late spring and falls off the branches after maturity. (Brown, 2001).

The Qatari culture is associated with some iconic desert trees such as arfaj (*Rhanterium epapposum,* it is play a significant role on the conservation of biodiversity,

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environmental sustainability. Arfaj is considered one of the main desert forage plants for camels and sheep.

Conservation of native plants are possible by their utilization in urban landscape, as indigenous plant species to the local harsh climatic conditions over the years and they are more likely to function adequately under the local climate as opposed to exotic plants (Suleiman *et al.,* 2009).

Conventionally, *Rhanterium epapposum* propagates through seeds, however harvesting and collecting seeds of plant is very difficult, particularly in arid regions where seeds can be confronted by severe environmental constraints such as high temperatures, drought and high levels of salinity as a result of fluctuating soil moisture (Brown, 2001).

Tissue culture plays a major role in overcoming the problems, which draws scientists, and researcher in the micropropagation of wild plants for rehabilitation of affected area Broun (2001). *In vitro* propagation techniques also enable preservation of the germplasm for plant species that are threatened with extinction (Gamborg and Phillips, 2013).

So far, there are no reports available on efficient micropropagation system for large-scale multiplication.

In this study, we describe a comprehensive culture method via organogenesis for conservation of the rare and endangered desert plant *Rhanterium epapposum* to expand its cultivation in dry areas and to be used as feed for animals.

Plant material and culture conditions

Commercial Micropropagation:

(i) **Initiation stage**

The current study was carried out in the Tissue Culture Laboratory of the Agriculture Research Department (A.R.D), Qatar. Plant material was obtained from the apical part of *Rhanterium epapposum.* The Apical Bud and nods used as explant materials were pre-washed with soapy water and kept under running water for one hour. Explants were surface-sterilized with 60% of commercial Clorox with 2 drops of tween -20 for 15 min under aseptic condition. Following by rinsed three times with sterilized distilled water, after that the explant were soaked in 20% of Clorox for 15 min and rinsed three times with D.W. Finally, dipped in 70% ethanol for few seconds and washed thoroughly in sterilized D.W.

Apical Buds and nods of *Rhanterium epapposum* cultured on full macro and duplication micro of modified Murashige and Skoog (1962) MS medium which supplemented with 100 mg l^{-1} myo-inositol , 120mg l^{-1} Adenine sulfate, 330 mg l^{-1} NaH_2PO_4, 1.5mg l^{-1} BAP, 30 g l^{-1} sucrose and 7gl^{-1} Agar. The PH of all media was Adjuseted to 5.7-5.8 and autoclaving (autoclaving at 121 ˚c, 1.2 kg cm^2 for 20 min). One explant was cultured in each magenta (culture box) containing 35 ml of medium. The cultured were incubated at 25±2°C in light with cool white fluorescent (16-h photoperiod day/night), the subculture could be conducted every four weeks for twice. Subsequently the cultures will shift to the multiplication medium to acquire desired number of shoots.

(ii) **Multiplication stage:** For mass propagation, developed shoots were cut into segments and cultured on full macro and duplication micro of modified MS medium supplemented with 100mg l^{-1} myo-inositol, 250 mg l^{-1} Adenine sulfate, 220 mg l^{-1} NaH_2PO_4, 1mg l^{-1} BAP, 0.5 mg l^{-1} kin, 30 gl^{-1}sucrose and 7 gl^{-1} Agar. All cultured explants were incubated at 25 ± 2ºC in light with cool white fluorescent (16-h photoperiod day/night) and sub-cultured every 3 weeks on same multiplication medium. Elongated shoots were detached from multiplication stage and subjected to rooting medium.

(iii) **Rooting stage:**

Elongated Shoots 3-5cm were selected from multiplication stage and cultured on MS medium with five different treatments in order to enhance the development of rooted plants.

1-Full MS+1 NAA
2-Full MS+1 IBA
3-1/2 MS+ 1 NAA
4-1/2 MS+ 1IBA
5-Full MS (control)

All media in this study composed of MS basal nutrient medium, supplemented with 0.22g/l mgl^{-1} NaH_2PO_4, 100 mgl^{-1}myo-inisitol and 30 gl^{-1}sucrose**.**

Each treatment containing three replicates and each replicate consists of five culture magenta with one of individual shoot. Culture were incubated at 25 ± 2ºC in light with cool white fluorescent (16-h photoperiod day/night (3000 lux). Data were collected after eight weeks about plant length, number and length of root.

Acclimatization stage:

Rooted plantlets were acclimate gradually to field condition by dipped in diluted fungicide solution for few seconds then transplanting in pots with a mixture of 1: 2:1 (sand, peat moss, perlite) and were covered with plastic. Potted plants were irrigated 10 times daily one minute at a time for 30 days. The plants were fertilized once a week with 2 g/l N-P-K (17-17-17) and fungicides.

After one month, remove the plastic gradually. Plantlets were transfer to greenhouse under the same conditions of irrigation (keep it for six month).

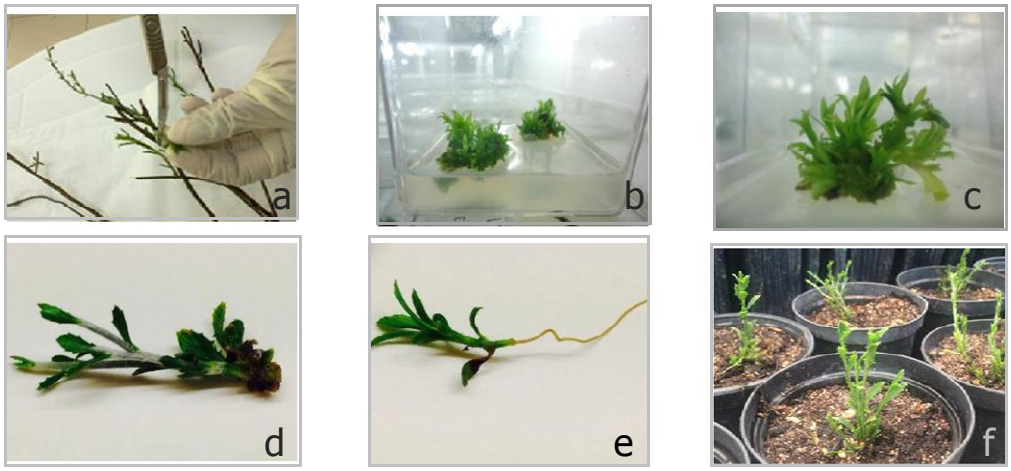

Figure 1: Stages of plant micropropagation of *Rhanterium* epapposum: a) initiation plant b,c) Shoot formation d) Shoot elongation e) Rooting plant f) plantlets in soil.

Statistical analyses

Factorial Randomized Complete Block Design with three replicates. Separation of means among treatments was determined using LSD test at 5% according to Steel *et al.* (1997)

RESULT AND DISCUSSION

In this study, we desirable commercial production protocol of *Rhanterium epapposum* (Fig.1). No study or literature reports on the commercial production used tissue culture technique.

Initiation stage: Apical Bud and nods cultured on full macro and duplication micro of modified MS medium supplemented with 1.5mg l^{-1} BAP will obtain shoots after8 weeks (Fig.1 a).

Multiplication and Elongation stage: Shoots were cultured on MS medium supplemented with BAP at 1mg l^{-1} and kin at 0.5 mg l^{-1}. After 8 weeks more shoots and buds were formed (Fig.1 b, c). Shoots were developed within three sub-culture to elongate shoots (Fig.1 d). Benzyladenine with kinetin founded more suitable for *Rhanterium epapposum* regeneration. PGRs play a vital role on in vitro plant growth and development, and the actions of auxins and cytokinins as promoters adventitious shoot development are well known. However, these effects depend on the type and concentration of the auxin and cytokinin and the ratio between them (George *et al*. 2008). Some tissues, organs or cell strains are able to grow without the incorporation of auxin in the medium; such cultures are termed auxin autonomous or auxin habituated (Machakova *et al*. 2008).

Rooting stage: The formation of root system *in vitro* for Arfaj plants is an essential step for them to survive after transplantation. Obtaining roots of Arfaj are very difficult *in vitro*, therefor the among goals of the study was to initiate ideally roots to surpass this problem.

Elongated shoots developed from multiplication stage had significant effects on all of the investigated traits. These are shown in Table 1 and Fig 2, the effect of different treatments on the plant length, number and length of roots of *Rhanterium epapposum*. Full-strength MS nutrient medium contained IBA enhanced the highest plant length (6.70 cm), root number (5.71) and root length (6.00 cm) at the concentration 1 mg l^{-1} (Fig.1e). On other hand Full-strength MS nutrient medium supplemented with NAA recorded less response in root formation. NAA at 1 mg l^{-1} gave plant length (4.70 cm), root number (4.50) and root length (3.70 cm). Full MS nutrient medium without growth regulators was poor in root formation. The highest plant length did not exceeded (1.20cm), root number (2.50) and root length (2.00cm).

Generally, auxin IBA more suitable to promote root initiation of *Rhanterium epapposum* than NAA, also full MS nutrient medium more effect than half MS to format roots.

On the contrary of our result, decreasing the level of salts in the medium means decreasing the level of nitrogen in the medium to half or quarter, this will result in decreasing nitrogen level in the shoots which may result in increasing the percentage of carbohydrates to nitrogen level and this may result in increasing the percentage of root primordial and roots number (Gawel *et al*., 1990). Shekhawat *et al*., 2015 reported that the *in vitro* generated shoots of *Passiflora foetida* were rooted on half-strength MS medium containing 2.5 mg l–1 indole-3 butyric acid (IBA). Also Shekhawat *et al*., 2014 reported that auxins (especially IBA) play an important role in the induction of roots from the cut ends of the in vitro raised shoots of *Salvia fruticose* and *Turnera ulmifolia*, respectively.

Table 1. Effects of the medium strength and plant growth regulators on the *in vitro* culture of *Rhanterium epapposum* after eight weeks of culture on rooting medium

Treatments mg l^{-1}	Average plant length (cm)	Average No. of roots	Average length of roots (cm)
1- Full MS+1 NAA	4.70	4.50	3.70
2- Full MS**+1** IBA	6.70	5.71	6.00
3-1/2 MS+ 1 NAA	4.70	5.00	3.00
4-1/2 MS+1 IBA	3.00	4.00	2.70
5-Full MS (control)	1.20	2.50	2.00
L.S.D	**3.71**	**3.82**	**3.00**

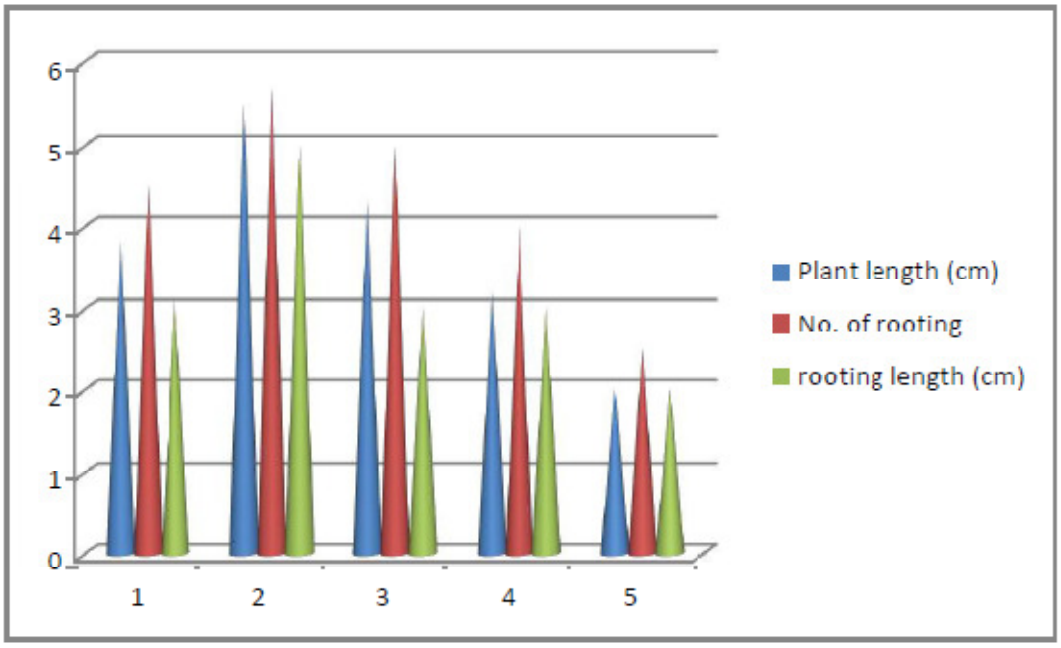

Figure 2: Effects of the medium strength and plant growth regulators on the *in vitro* culture of *Rhanterium epapposum* after eight weeks of culture on rooting medium

These results proved that auxins have a role in rooting process since they promote adventitious roots initiation in the bases of cultured shoots (Abdul, 1987 and Saleh, 1990). Bader *et a*l (2000) endogenous hormones might have a role in promoting plants to root (Peak *et al*., 1987), until the hormonal balance reached its optimal level to push the roots to grow and develop in the presence of exogenous hormones, since increasing of auxins concentration promotes root formation on shoots bases (George and Shermington,1984). Al-Khateeb 2008b mentioned that root formation required more energy than bud or shoot formation. Various growth regulators influence in different way on root induction, elongation, and biomass production (Gyulai *et al*., 1993; Biondi *et al.* 1997; Bålåvanyos *et al*., 2001; Balestri and Bertini, 2003).

Biradar *et al.,* 2009 mentioned that higher concentrations of NAA is inhibitory both to root induction and elongation in sugarcane. Increased concentrations of auxins stimulate natural ethylene production, which is inhibitory to rooting. However, despite many reports on the physiological action of both phytohormones, the molecular mechanisms of their effect(s) on cell expansion, cell division, differentiation, organogenesis, and the mechanisms of their interactions have not yet been elucidated George et al. (2008).

Plantlet acclimatization e

The ultimate success of plant tissue culture depends on transplanting the plantlets successfully to soil. After transfer from the *in vitro* to the *ex vitro* condition, plantlets need gradual changes in environmental conditions to avoid desiccation losses and photo inhibition (Kozai *et al*., 1991). Plantlets were survived 65% after six months of acclimatization in greenhouse (Fig. 1f).

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