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In Vitro Plant Regeneration of Libyan Wild Plants: Edible Species (Arbutus pavarii) and Endanger Species [Haplophyllum tuberculatum (Forsk.) Juss]

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ABSTRACT: *Protocol of micropropagation of* ***Arbutus pavarii*** *and* ***Haplophyllum tuberculatum*** *was successfully achieved.* ***In vitro*** *plant regeneration of* ***Arbutus pavarii*** *was attempted using single nodal on MS medium supplemented with different concentration of three various growth regulators indole-3-butyric acid (IBA), Kinetin (K) and N-Isopentenylaminopurine (2ip). Multiple shoots from single node were obtained on MS medium with the 0.5 mg/l 2ip. While, faster improving of shoots elongation was on MS contained 0.5 mg/l K. Explants obtained in micropropagation step were used for rooting step under several treatments. The best results were obtained when explants were sup-cultured on MS medium with 1 mg/l IBA. New plants were vigorous, of good quality and presented phenotypic characters similar to mother plants. Micropropagation of* ***Haplophyllum tuberculatum*** *was achieved from sterilized single nodal segments on MS medium supplemented with for different concentrations of 2,4-D for callus induction and other different hormones K and BA for multiplication of auxiliary branches and rooting . The highest results of the weight of callus were growing in MS medium containing 1 or 2 mg/l 2.4-D hormone with maltose. Whereas, the axillary soothing was significantly proliferated on MS medium supplemented 2 mg/ l^{-1} K. Acclimation of plantlet was in greenhouse.*

Keywords: Micropropagation, growth regulators, ***Arbutus pavarii****,* ***Haplophyllum tuberculatum****,* ***in vitro*** *culture.*

INTRODUCTION

Plant biodiversity is reported that one of important genetic resources of many species growing spontaneously around the Mediterranean basin (Louhaichi *et al*, 2011). Libya is one of Mediterranean basin country and a native of many plant species that model of biodiversity (El-Darier and El-Mogaspi, 2009). Plants that have economic importance such as *Haplophyllum tuberculatum* (Forsk.) Juss and strawberry trees (*Arbutus pavarii)* which are located in different environments (coastal, mountainous and desert) in Libya (El-Darier and El-Mogaspi, 2009). There are a number of factors for the difficulty of germination and growth of these plants naturally in the wild land. Drop in rate of rainfall annually it is the most important environmental factor which has made the wild lands drier and decreased significantly seeds germination (Elmaghrabi *et al.,* 2017). In addition, overgrazing and the use of lumber as firewood and also expansion of new farms, which contributed of deterioration sharply of edible and medical wild plant resources which led these species to endanger (Elmaghrabi *et al* 2017).The genus *Arbutus* belongs to the *Vaccinioideae* subfamily which includes evergreen shrub-like woody taxa with laurel-like and sclerophyllous leaves of the *Ericaceae* family (Torres *et al*., 2002). There were about six species

of *Arbutus* grows spontaneously around the Mediterranean basin. The species is drought tolerant and able to regenerate following forestry fires making it quite interesting for forestation programs in Mediterranean regions. Fruits are used to make jellies and a spirit which represents the main income for owners (Torres *et al*., 2002). *Arbutus pavarii* species it is only located in the coast of Libya especially at El-Jabal El-Akhdar Region in the Mediterranean Regions (El-Darier and El-Mogaspi, 2009). *Haplophyllum tuberculatum* (Forsk) juss that belongs to the Rutaceae familly (El-Naggar *et al*., 2014). *Haplophyllum tuberculatum* is a herbal plant is, simple leaves, reciprocal, heterogeneous white, small yellow flowers (Puricelli *et al*., 2002). The whole plant is being used in pharmaceutical product with the exception of the roots. The essential oil of *Haplophyllum tuberculatum,* which prepared by hydrodistillation of the fresh flowering aerial parts of the plant collected from wild types (Al-Rehaily *et al*., 2014). The oil was subsequently analyzed by GC and GC-MS. Thirty seven compounds, accounting for 96.4 % of the oil composition were identified in this study (Al-Rehaily *et al.*, 2014). The antimicrobial and activity of the essential oil was also evaluated against various human pathogens, where a relatively low inhibitory range was observed. Because these species (*Haplophyllum tuberculatum* and *Arbutus pavarii*) which have good economic value and, the scarcity of plant biomass available in the natural habitat, we set up non-conventional methods for plant propagation from nodal stem segments and, at the same time, we established cell cultures of the plant (Elmaghrabi *et al.*, 2017). Native plants, calli and suspension cultures were found to produce several plant regeneration which was true-to-typeness (Elmaghrabi and Ochatt, 2006). Micropropogation of endanger and medical plants which difficult to propagate under normal condition for biodiversity and environmental balances that will lead to improve agriculture facility in Libya. The purpose of this study was to develop an efficient protocol for *in vitro* propagation of endanger edible strawberry tree (*Arbutus pavarii*) and medical plant *Haplophyllum tuberculatum*.

MATERIALS AND METHODS

In order to investigate the effects of different concentrations of growth regulator into MS medium (Murashige and Skoog, 1962) for *in vitro* culture initiation and callus formation. Single nodal segments were used as explants, obtained from young growth of an adult plant growing in the wild near Tripoli for *Haplophyllum tuberculatum* and El-Jabal El-Akhdar Region for *Arbutus pavarii.* Following excision, all explants were well washed under running tap water. Explants were surface-sterilized with immersed in ethyl alcohol concentration of 70% for two minutes and with 20% (v/v) commercial sodium hypochlorite (NaOCl) with 0.1% Tween 20, for 15 min, followed by four 3-min rinses with sterile distilled water. Explants of *Arbutus pavarii* placed (25×100 mm, one explant per tube) with 10 ml medium. The test tubes were covered with transparent plastic film. The culture medium consisted of MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar. The effect of three growth hormones at 0.5 or 1.0 mg/l each was compared: benzyladenine (BA), kinetin (K) *N*-isopentenyl adenine (2iP) or indole-3-butyric acid (IBA). While the four explants of *Haplophyllum tuberculatum* were placed in Petri dish with 20 mL MS medium with 3% (w/v) maltose supplemented various concentration (0.5, 1.0, 2.0 or 4.0 mg/l) of 2,4-Dichlorophenoxyacetic acid (2,4-D) for callus induction. Then the callus were sub-cultured on MS medium with different concentration (0.1, 0.5, 1.0 or 2.0 mg/l) of K or BA. Forty-eight explants for both species (*Arbutus pavarii* and *Haplophyllum tuberculatum*) per treatment were used. After 45 days in culture, data were recorded evaluating shoots length and number of: branches, leaves and roots and a subculture were carried out using the media of the initial culture of each three weeks. Cultures were incubated at 25°C, 16 h photoperiod under 37.5

μmol m^{-2} s^{-1} fluorescent light. The pH of the media was adjusted at 5.6-5.7 before autoclaving at 121°C for 20 min. For ex vitro acclimatization well rooted micro shoots were transferred to 300 ml jars, containing 1 part of perlite and 1 part of compost (v/v). The plants, for both species initially covered with transparent film, and placed under, 16-h photoperiod (natural day light extended with incandescent light). The completely randomized design was used. The significance of the results was tested by ANOVA with Minitab software (version 17) and the means were compared by Student's t at P=0.05.

The percentage of responding explant did not exceed 79% for *Arbutus pavarii*. However, cultures were successfully established at intervals of 45 days (Table 1, Fig 1). The best results of root number (24.2 per explant) were obtained when explants sup-cultured on MS medium supplemented with 1 mg/l IBA (Table 1). The shoot elongation (2.96 cm) and the number of roots (10.8) were the high medium on supplemented with 0.5 mg/l K, comparison to media with BA or 2iP (Table 1).While, on medium with 0.5 mg/l 2ip produced the highest number of leaves (12.7) and number of branches (2.0) (Table 1).0.5 mg/l of K or 2ip was much more effective for shoot length, root induction and number of: branches and leaves than BA or IBA or even 1 mg/l K or 2ip which were produced the lowest number of roots 2.9 and 2.8, respectively (Table 1). Therefore, K or 2ip at 0.5 mg/L proved the most effective cytokinin for culture initiation, multiplication and acclimatization *in vivo* culture stages of *Arbutus pavarii* (Fig 1).

Similar results concerning the effectiveness of various cytokinins on shoot production have been reported for *A. andrachne* (Bertsouklis and Papafotiou, 2009). It is reported that in plants of the family *Ericaceae*, the natural compounds zeatin and 2iP are more effective than other cytokinins for shoot proliferation (George *et al*., 2008). Rooting is essential to the success of micropropagation. Without an effective root system plant acclimatization will be difficult and the rate of plant propagation may be severely affected (Gonçalves *et al*., 1998). Seventy-five of the micro shoots were rooted *in vitro* on MS with 0.5 mg/l K and 70% of the micro plants established successfully acclimatization *in vivo* culture (Fig 1).

In terms of *Haplophyllum tuberculatum,* during assessment stage of callus induction on MS medium supplemented with four various concentration of 2,4-D hormone, 2 or 4 mg/l 2,4-D was produced significantly the highest weight of callus induction, in comparison of 0.0, 0.5 or 1.0 mg/l 2,4-D (Fig. 2). Amongst two cytokinins tested, K proved to be more effective than BA for initiating shoots and a higher yield of shoot per explant (Table 2). Kinetin at 1mg/l seemed to be the best concentration, since it facilitated a high rate of proliferation, shoot induction, number of leaves and shoot elongation (Table 2). Although at a higher concentration, K (2 mg/l) stimulates a biggest of number of brunches, but significantly reduced their shoot length, number of roots and also associated with shoot induction (Table 2). Conversely BA (1-2 mg/l) induced limited average of shoot induction, but stimulated number of branches. While, medium with 2 mg/l produced significantly the best number of roots (Table 2). The shoot induction and root development was significantly decreased when callus sub-cultured on MS medium supplemented with 0.1 mg/l for both cytokinins K and BA. Whereas, 0.5 mg/l BA produced high of shoot length and number of branches, but 0.5 mg/l K it is only stimulated shoot elongation (Table 2). Therefore, micropropagation of *Haplophyllum tuberculatum* was achieved and all stages *in vitro* culture exhibited in this study started from callus induction on MS medium with 2 mg/l 2,4-D until plantlet acclimatization *in vivo* culture (Fig 3). According to earlier articles the aptitude for proliferations of this medical plant species was limited. In comparison although similar results were reported on B5 medium supplemented with 1.3 mg/l 2,4-D and 0.25 mg/l K (Puricelli *et al*, 2002). Protocol described in this study with 1mg/l K involved five sub-cultured resulting in higher yield of axillary shoots. Moreover, the rooting and shooting rates reported here is higher than one previously obtained by Gholami *et al*. (2009) on MS medium supplemented with 1 mg/l IBA. *In vitro* rooting

and ex vitro establishment were similar to that reported for *A. andrachne* (Bertsouklis and Papafotiou, 2009). Similarly, *Capparis spinosa L.* plantlets obtained *in vitro* were easily acclimatized (Elmaghrabi *et al.*, 2017).

CONCLUSION

These results indicate the enormous potential of these methods for the biomass propagation of both species *Arbutus pavarii* and *Haplophyllum tuberculatum*.

The protocols developed in this study will be useful for micropropagation of endanger and medical plants which difficult to propagate under normal condition for biodiversity and environmental balances that will lead to improve agriculture facility in Libya.

Table 1. The effects of growth hormones on the percentage of explants responding, the number of: branches, leaves, roots and shoot length, per explants of *Arbutus pavarii* after 45 days of culture initiation of single nodal sections on MS medium.

Treatment (mg L^{-1})	Shoot length (cm)	Number of branches	Number of leaves	Number of roots	Explants responding (%)
Control (0)	1.48	0.93	4.1	0.1	18.2
0.5 BA	1.52	1.50 *	9.6 *	4.8 *	32.7 *
1.0 BA	1.92	1.60 *	9.9 *	3.7 *	58.2 **
0.5 2ip	2.07 *	2.00 *	12.7 *	7.7 **	68.8 ***
1.0 2ip	2.01 *	1.60 *	9.9 *	2.8 *	64.7 **
0.5 K	2.96 **	1.70 *	11.9 *	16.8 ***	75.8 ***
1.0 K	1.99	1.80 *	12.2 *	2.9 *	77.5 ***
1.0 IBA	2.36 *	1.10	7.8	24.2 ***	72.8 ***

Means of the same column which are followed stars are statistically different at $p \leq 0.05$.

Table 2. The effects of cytokinin on the average of shoot induction, the number of: branches, leaves, roots and shoot length, per explants of after 45 days of culture initiation of *Haplophyllum tuberculatum* Forsk. Juss callus of single nodal sections on MS medium.

Treatment (mg L^{-1})	Shoot length (cm)	Number of branches	Number of leaves	Number of roots	Average of shoot indication
Control (0)	1.0	3.0	1.1	0	17.1
0.1 BA	1.3	4.0	1.2	0	50.2 **
0.5 BA	1.8 *	14.0 *	4.5 **	2.9 **	62.0 ***
1.0 BA	1.7 *	15.1 **	4.4 **	3.2 **	13.3
2.0 BA	1.4	13.0 *	4.2 **	5.3 ***	17.1
0.1 K	1.2	9.0	1.0	0.1 *	13.0
0.5 K	1.9 *	12.1 *	2.6 *	0.9 *	25.2 *
1.0 K	2.0 *	16.2 **	4.1 **	4.5 ***	73.0 ***
2.0 K	1.4	14.1 *	5.3 **	1.7 *	54.4 **

Means of the same column which are followed by stars are statistically different at $p \leq 0.05$.

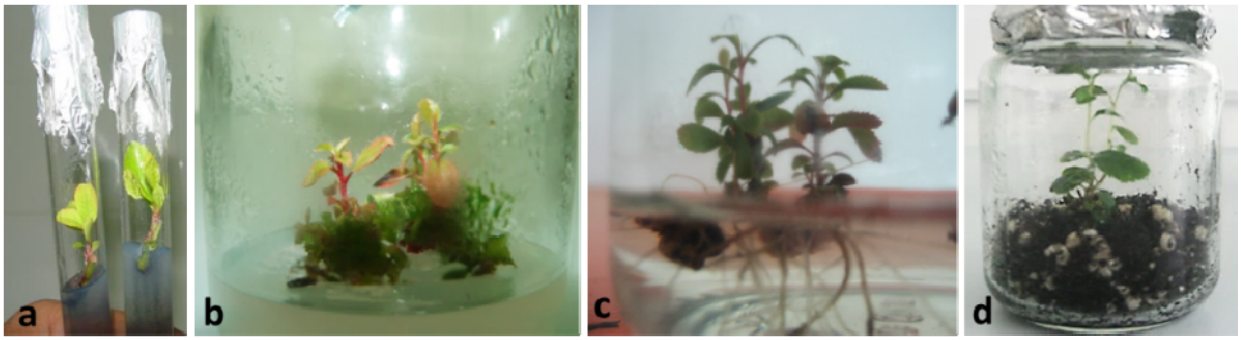

Figure 1. Stages of micropropagation of *Arbutus pavarii*; a, Shoot induced from nodal segments on modified MS medium supplemented with 0.5 mg/l K; b, Multiplication of shoots; c, Four week old rooted shoots on same medium (0.5 mg/l K); b, *Ex vitro* acclimatization well rooted micro shoots were transferred to 300 ml jars, containing 1 part of perlite and 1 part of compost (v/v) for full plant.

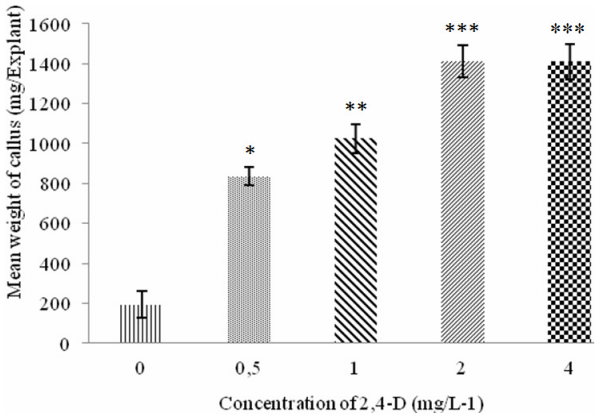

Figure 2. Composite mean (± S.E.) callus growth of *Haplophyllum tuberculatum* Forsk. Juss after a four-week on MS medium supplemented with four various concentrations (0.5, 0.1, 2.0 or 4.0 mg/l) of 2,4-D. Callus FW was recorded after four weeks. ($P \leq 0.05$) (n= 12 replicates).

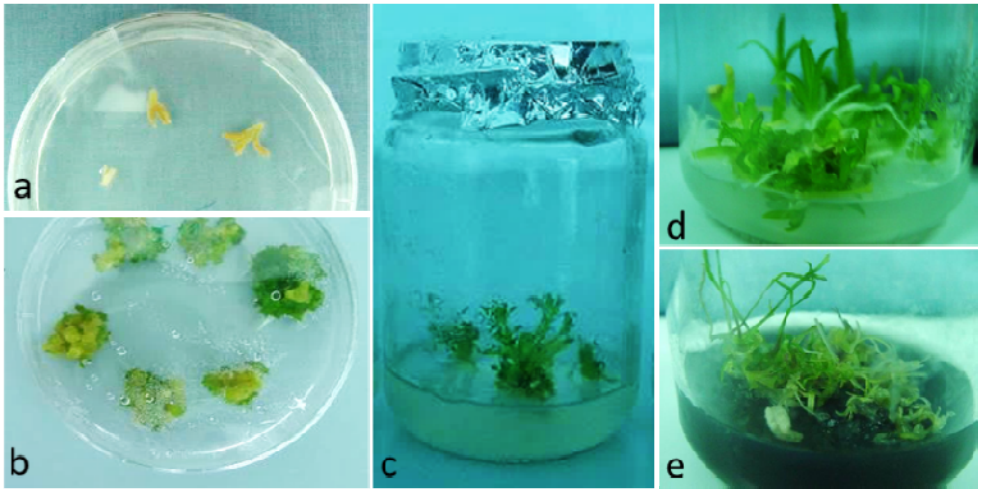

Figure 3. Stages of micropropagation of *Haplophyllum tuberculatum* Forsk. Juss; a, Single nodal segments; b, Callus induction and development on modified MS medium supplemented with 2 mg/l 2.4-D; c, Multiplication of shoots on MS medium contained 1.0 mg/l K; d, Six week old rooted shoots on same medium (1.0 mg/l K); e, Ex vitro acclimatization well rooted micro shoots were transferred to 300 ml jars, containing 1 part of perlite and 1 part of compost (v/v) for full plant.

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