IN VITRO PROPAGATION AND SECONDARY METABOLITES PRODUCTION IN THE WILD RARE *ASPARAGUS APHYLLUS* L. PLANT

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Asparagus aphyllus is grown in Egypt naturally as a rare plant. It is utilized in conventional medicine to treat various liver diseases and as anti-cancer. Shoot tips have been cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l kinetin (kin) and 6-benzyl adenine (BA) combined with different concentrations of α-naphthalene acetic acid (NAA) for the multiplication of shoots. The shoot of 5 cm has been cultured on 1/2 MS medium supplemented with various concentrations of indole-3-butyric acid (IBA) or full MS medium with 1.0 mg/l kin and two concentrations of NAA for rooting. The top results of multiplication were obtained with 1.0 mg/l BA and 0.2 mg/l NAA and gave 12 shoots per explant and the maximum mean length of shoot was 6.60 cm with 1.0 mg/l BA and 0.4 mg/l NAA. The highest percentage of rooting was 20% after three months of culture on half strength MS medium with IBA at 1.5 mg/l and 2.0 mg/l. The mean number of roots per explant was 1.6 roots. The mean length of root was 4.0 cm at a concentration of 2.0 mg/l IBA. Percentage of survival after acclimatization was about 65% after two months. The highest fresh weight of callus was 14.03 g with friable texture and observed on MS medium supplemented with 2.0 mg/l NAA plus 1.0 mg/l 2.4- D with 0.5 mg/l BA. The total saponin that contains the highest value of 5.85 mg/g fresh weight was observed from the mother plant. The results of the present study would be applicable for cost-effective large-scale micropropagation of *Asparagus aphyllus* and improvement.

Keywords: Asparagus, *in vitro*, micropropagation, saponin

INTRODUCTION

Asparagus aphyllus L. (Prickly), is used for human food, conventional medicine and in treating various disease (Knaflewski, 1996). It is known as Asparagus in English and Halion in Arabic, and is exercised in classical Greco-Arab and Islamic medicine (Saad and Said, 2011). The genus *Asparagus* includes around 200 species and classified into three subgenera. *A. aphyllus* is adapted to dry conditions and in hedges and scrub as well as rocky areas and crevices (Turland et al*.,* 1993). *A. aphyllus* is native to southern Europe, as well as north Africa (Egypt) and Jordan, Lebanon, Syria and Turkey in western Asia (USDA, ARS, National Genetic Resources Program, 2014). *A. aphyllus* is a secondary genetic relative of *Asparagus* (*A. officinalis*) (USDA, ARS, National Genetic Resources Program, 2013) and so it has a potential for use as a gene donor for crop improvement, especially as it is adapted to xerophytic conditions and is resistant to diseases (Falavigna et al., 2008). According to Tardio et al. (2006), young shoots are harvested and eaten with eggs as an omelette in Spain. Kubota et al. (2012) reported that the geographic distribution of these species comprises the arid and semiarid regions of Europe, Asia, Africa and Australia. All the species in the subgenus *Asparagus* are dioecious as reported by Castro et al. (2013).

Phytochemicals from the root of *Asparagus* (dioscin and methylprotodioscin) were reported *in vitro* to inhibit the expression of mucin protein in the epithelial cells, which partially validates traditional uses in inhibiting hypersecretion in the mucus of pulmonary tracts (Lee et al., 2015). The plant treat varied liver diseases, diarrhea and dysentery (Saad and Said, 2011). Meanwhile, other studies have reported that *Asparagus* gum polysaccharide have immune-modulatory functions and were shown to accelerate apoptosis, diminishing tumor development of hepatocellular carcinoma in the liver. Cladophyll and roots of *Asparagus in vivo* lower diastolic blood pressure, glucose, left cardio-ankle vascular index score and total cholesterol level (Nishimura et al., 2013 and Weng et al., 2014). *A. aphyllus* is used as anti-cancer (Kmail et al., 2015), inhibitor for the growth of bacteria (Teka et al., 2015) and a rich source of antimicrobial agents (Sharma and Goel, 2018). Kmail et al., (2017) reported that the extracts of *A. aphyllus* exhibit relatively high levels of phenolics, flavones and flavonols.

The *Asparagus* genus is one of the few plant foods containing steroidal saponins that are distributed throughout different organs of the plant, including leaves, stems, fruits and roots (Hamdi et al., 2017). As regard to the saponins, they are remarkably stable to heat processing, and their biological activity is not reduced by normal cooking (Kimura et al., 2006). Saponins are essential constituents of nutraceuticals and useful foods (Raju and Mehta, 2009) and are recognized with a number of bioactivities like anti-inflammatory, antimicrobial, immunostimulant, hypocholesterolaemic, anticarcinogenic and antioxidant (Guclu-Ustundag˘ and Mazza, 2007). In addition, saponins form complexes with cholesterol of erythrocytes membrane forming pits and holes, which leads to the increase in permeability and haemolysis (Hostettmann and Marston, 1995).

There are a few researches about micropropagation of *A. aphyllus*. The use of *in vitro* propagation methods is necessary to propagate *A. aphyllus* through different micropropagation procedures as reported by Desjardins et al. (1987) and Kunitake and Mii (1998). However, a general protocol is valid for all the species of *Asparagus*. The main purpose of the present study was to optimize an *in vitro* propagation method for *A. aphyllus* and the *in vitro* production of saponins.

MATERIALS AND METHODS

1. *In Vitro* Propagation

1.1. Plant material and sterilization

The seeds of *A. aphyllus* were collected in August 2019 from Al-Arish region and utilized as a source of plant material. Seeds were surface sterilized under running tap water for three hours with a few drops of soup, then transferred to a laminar air flow cabinet to complete sterilization by sodium hypochlorite solution (5.25%) (commercial bleach 15%) for 5-10 min. After that, seeds were rinsed with sterile distilled water for three times and cultured

in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), then incubated in the culture room at 23±2ºC in complete darkness, for one month for germination.

1.2. Induction and multiplication of shoots

Shoot tips were excised from the seedlings after one month and cultured on MS medium supplemented with kinetin (kin) or 6-benzyl adenine (BA) at a concentration of 1.0 mg/l combined with 0.2, 0.4 or 0.6 mg/l α-naphthalene acetic acid (NAA) for the multiplication stage. The mean number of the shoots and mean length (cm) were counted after 30 days of culture for each treatment.

1.3. Rooting stage

The multiplied cluster (5 cm long) were inoculated into ½ MS medium supplemented with 30 g/l sucrose, 2.5 g/l phytagel and 1.0, 1.5, or 2.0 mg/l of indole-3-butyric acid (IBA) or full MS medium with 1.0 mg/l kin with 0.4 or 0.6 mg/l NAA for rooting.

1.4. Acclimatization stage

Healthy rooted clusters were transferred into small pots (10 cm in diameter) containing 1:1:1 (v/v/v) sand: peat: perlite under greenhouse conditions. The pots were covered with polyethylene bags for 5 weeks to maintain humidity. Then plantlets were transferred into large pots for 3-4 months.

2. *In Vitro* Production of Saponins

2.1. Induction of callus

Stems cuttings were cultured on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at different concentrations or NAA with BA at 0.5 mg/l and 2.0 mg/l 2,4-D or 2.0 mg/l NAA with 1.0 mg/l kin for the initiation of callus. The mean fresh weight, texture and color of callus were measured after 60 days of culture in complete darkness for each treatment.

2.2. Extraction and determination of saponins

The determination of total saponins was done by the standard method of Obadoni and Ochuko (2002) with minor modifications. Callus (1 g) was added to 100 ml of 20% aqueous ethanol and kept in a flask on a stirrer for half an hour, then heated for 4 h at 45°C with mixing. The mixture was filtered by using Whatman filter paper no. 1 and the residue again extracted with another 100 ml of 25% aqueous ethanol. The combined extracts were concentrated using a rotary evaporator in 40°C to get 40 ml approximately. The concentrate was transferred into a separation funnel and extracted twice with 20 ml diethyl ether. The ether layer was discarded while the aqueous layer was kept and then re-extracted with 30 ml n-butanol. The n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated. After evaporation, the samples were dried in the oven at 40°C to a constant weight and the saponin content was calculated as a percentage:

Total yield of saponins (%) = (Weight of saponins / Weight of sample) x100.

3. Statistical Analysis

Each experiment was set up as a completely randomized design. Data were tested according to the analysis of variance (ANOVA) using Costat statistical package software. Means were separated according to the least significant difference (LSD) test at the 0.05 level probability

RESULTS AND DISCUSSION

In vitro propagation, rooting and callus induction of *A. aphyllus* on MS medium supplemented with different concentrations of growth regulators were evaluated.

1. *In Vitro* Propagation

1.1. Induction and multiplication of shoots

Shoot tips of seedlings were cultured on MS medium plus different concentrations of cytokinins and NAA. Data in table (1) show the effect of cytokinins and NAA on the number and length of *A. aphyllus* shoots. Data in table (1) show that the highest value for the mean number of axillary shoots per explant was observed in explants cultured on MS medium supplemented with 1.0 mg/l BA and 0.2 mg/l NAA and were 12 shoots. The explants cultured on MS medium plus 1.0 mg/l kin and 0.6 mg/l NAA significant had the lowest number compared with the other treatments. While, the highest mean length of axillary shoots (6.60 cm) was obtained with 1.0 mg/l BA and 0.4 mg/l NAA. It was observed that BA with high concentrations of NAA formed callus. It was noticed that BA was superior in multiplication in comparison with kin (Fig. 1A).

Table (1). Effect of cytokinin and NAA on shoot multiplication of *Asparagus aphyllus.*

Concentrations (mg/l)		Mean number of axillary shoot / explant	Mean length of axillary shoot (cm)	Callus formation
1.0 kin	0.2 NAA	8.25^{a-c}	5.56^{a}	-
	0.4 NAA	7.25^{a-c}	5.31^{a}	-
	0.6 NAA	3.25^{c}	4.45^{a}	-
1.0 BA	0.2 NAA	12.00^{a}	5.31^{a}	-
	0.4 NAA	4.00^{bc}	6.60^{a}	+++
	0.6 NAA	9.00^{ab}	5.67^{a}	++

Data are expressed as mean columns by the LSD test and different lowercase letters indicate significant difference at a 5% level

Similar results were found with the best shooting on MS medium supplemented with kin and BA on *Asparagus racemosus* by Pandey et al. (2016). While, the highest shoot multiplication was achieved with MS medium enriched with BA at 0.1 mg/l plus NAA and 0.05 mg/l as concluded

on *A. racemosus* by Patel and Patel (2015). While, Pant and Joshi (2018) noted that *A. racemosus* gave adventitious shoots on low concentration of IBA in combination with relatively higher concentrations of kin in MS medium. Among the treatments where hormones were tested singly, IAA at 0.5 mg/l and kin at 1.0 mg/l, Paudel et al. (2018) found that MS medium supplemented with 0.5 mg/l BA and MS medium plus NAA at 0.5 mg/l and kin at 1 mg/l gave the most significant shoot multiplication of *A. racemosus*. The interactions between endogenous plant growth regulators in varied plant organs at assorted developmental stages are very complex, and the manner in which a given explant will react to various concentrations of diverse exogenous growth regulators *in vitro* can vary greatly depending on the species, variety, age, and source of the explants, according to Pollard and Walker (1990).

1.2. Rooting stage

Data in table (2) indicate that there is no significant difference between the treatments and all the parameters recorded. The highest percentage of rooting per explant was 20% after three months of culture, when the explants cultured on ½ MS medium with IBA at 1.5 mg/l and 2.0 mg/l. The mean number of roots per explant was 1.6 roots. The mean length of the root was 4.0 cm at 2.0 mg/l IBA. It was noticed that all concentrations of IBA formed callus. These results were given by Patel and Patel (2015), who reported that *in vitro* shoots of *A. racemosus* were cultured on ½ MS basal medium and rooting was observed with IBA at 1.5 mg/l in nodal explants. While, Afroz et al. (2010) found that the shoots of *A. racemosus* were rooted best on ½ MS medium amended with 0.05 mg/l BA combined with 1.0 mg/l IBA.

Table (2). Effect of ½ MS medium with IBA on rooting of *Asparagus aphyllus* after 3 months.

IBA concentration (mg/l)	Percentage of rooting (%)	Mean number of roots / explant	Mean length of root (cm)	Callus formation
0.0	0	0.0	0.0^{b}	-
1.0	0	0.0	0.0^{b}	++
1.5	20	1.6	2.5^{ab}	++
2.0	20	1.6	4.0^{a}	++

Data are expressed as mean columns by the LSD test and different lowercase letters indicate significant difference at a 5% level

The important component was IBA, it was regulating root regeneration and is considered more potent than other auxins (Ludwig-Muller,

2000). IBA induces and enhances rooting quality (Hartmann et al., 2002). Root induction is dependent on the presence of IBA, whether endogenous or artificially applied (Haissig, 1972). In addition, cuttings treated with IBA stimulates a more uniform root to produce (Hartmann et al., 2002). However, the synthesis of rooting can significantly affected by IBA concentration (Raven et al., 1999). The effect of IBA on the rooting synthesis may be due to its effect on cell wall turgidity, which accelerates cell division (Hartmann et al., 2002).

Data of table (3) show clearly no significant differences among treatments. It was observed that the best treat was MS medium containing 1.0 mg/l kin in combination with 0.6 mg/l NAA, the mean number of root per explant was given 1.6 roots per cluster and the mean length of the root was 4.0 cm, and all treatment of kin plus NAA formed callus on explants (Fig. 1B). Regalado et al. (2015) reported that more than 70% of *Asparagus* developed shoots and the rooting rate on MS medium was 30–45%. The rooting rate increased to 60–85% when the unrooted shoots were subjected to an additional cycle of rooting, reaching 100% after two cycles of rooting.

1.3. Acclimatization stage

Healthy rooted clusters were successfully transferred to a sterilized mixture of 1:1:1 v/v/v sand: peat: perlit for three weeks in a growth chamber, then gradually acclimatized in the greenhouse with a percentage of survival of about 65% after two months (Fig. 1c).

Table (3). Effect of MS medium with kin and NAA on rooting of *A. aphyllus,* data were recorded after 6 months.

Concentrations (mg/l)	Percentage of explant root (%)	Mean number of roots / explant	Mean length of root (cm)	Callus formation
0.0	0	0.0 ^{b}	0.0^{b}	
1 kin+0.4 NAA	25	1.6 ^{a}	3.17^{a}	+++
1 kin+0.6 NAA	25	1.6 ^{a}	4.00^{a}	++

Data are expressed as mean columns by the LSD test and different lowercase letters indicate significant difference at a 5% level.

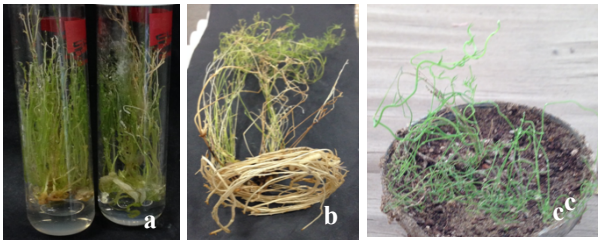

Fig. (1). *In vitro* propagation of *Asparagus aphyllus*; **(a)** multiplication of shoots **(b)** rooted plantlet, and **(c)** acclimatization of transplants in the greenhouse.

2. *In Vitro* Production of Saponins

2.1. Induction of callus

It could be observed from data in table (4) that MS medium supplemented with different concentrations of NAA and/ or 2,4-D and 0.5 mg/l BA initiated callus (Fig. 2a and b). Data indicate that the highest fresh weight of callus was observed on MS medium supplemented with 2.0 mg/l NAA plus 1 mg/l 2,4- D with 0.5 mg/l BA, it gave 14.03 g callus with friable texture and piege color, followed by 2.0 mg/l NAA plus 2.0 mg/l 2,4- D with 0.5 mg/l BA and 2.0 mg/l NAA only that gave 8.96 and 7.43 g, respectively, the texture of callus was compact and piege with green color. The lowest weight was observed at concentration of 2.0 mg/l 2,4- D only, it gave 0.33 g. Some treatments formed direct organogenesis, which were 1.0 mg/l 2,4-D, 1.0 mg/l 2,4-D + 0.5 mg/l BA, 1.0 mg/l NAA +1.0 mg/l 2,4-D + 0.5 BA mg/l and 1.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l BA). On the other hand, Pise et al. (2012) found that in *A. racemosus* the MS medium supplemented with 1.0 mg/l NAA plus 1.0 mg/l 2,4-D with 0.5 mg/l BA gave the highest weight of callus and percentage of callus initiation. Also, Patel and Patel (2015) found that the highest percentage of callus induction of *A. racemosus* was observed in MS medium supplemented with 0.1 mg/l NAA.

Table (4). The effect of MS medium supplemented with different concentrations of NAA and /or 2.4 D and /or 0.5 mg/l BA on callus induction of *A. aphyllus*

Concentrations (mg/l)			Callus formation (%)	Callus fresh weight (g)	Texture	Colour
NAA	2,4-D	BAP				
0	0	0.0	0	--	-	-
1	0	0.0	100	3.73^{b-d}	Compact	Bage
2	0	0.0	100	7.43^{a-c}	Compact	Piege +
1	0	0.5	30	4.73^{b-d}	Compact	Green
2	0	0.5	60	4.23^{b-d}	Compact	Piege +
0	1	0.0	0	-	Organogenesis	-
0	2	0.0	30	0.33^{cd}	Compact	Piege
0	1	0.5	0	-	Organogenesis	-
0	2	0.5	20	0.73^{cd}	Compact	Piege
1	1	0.5	0	-	Organogenesis	-
2	1	0.5	100	14.03^{a}	Friable	Piege
1	2	0.5	0	-	Organogenesis	-
2	2	0.5	100	8.96^{ab}	Compact	Piege +
	LSD 0.5			8.43		

Data are expressed as mean columns by the LSD test and different lowercase letters indicate significant difference at a 5% level.

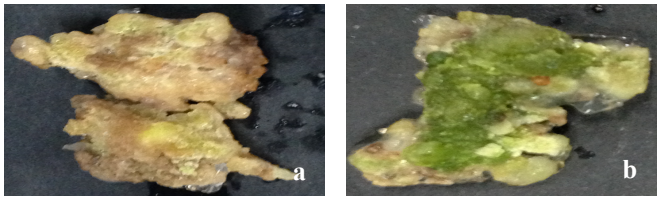

Fig. (2). Induction of callus of *Asparagus aphyllus*; **(a)** MS medium plus 2,4-D **(b)** MS medium plus NAA.

2.2. Saponin content

Data in table (5) show the total saponin content in the mother plant, shootlet and callus of *Asparagus aphyllus.* The total saponin that had the highest value was observed in the mother plant and callus cultured on MS medium supplemented with 1 mg/l kin plus 2 mg/l 2,4-D, this medium followed by that supplemented with 1 mg/l kin plus 2 mg/l NAA was 5.85, 5.74 and 5.67 mg/g fresh weight, respectively. Pise et al. (2012) found that in *A. racemosus*, MS medium supplemented with 1.0 mg/l NAA plus 1.0 mg/l 2,4-D with 0.5 mg/l BA gave the maximum level of total saponin and biomass accumulation. Biomass and saponin accumulation patterns depended on the combinations of growth regulators and the pH of the medium.

Table (5). Total saponin content (TSC) in mother plant, shootlet and callus of *A. aphyllus*.

Treatments	TSC mg/g
Mother plant	5.85
Shootlet	5.55
Callus (1 mg/l kin+ 2 mg/l 2,4-D)	5.74
Callus (1 mg/l kin+ 2 mg/l NAA)	5.67

CONCLUSION

The results of the present study highlight a fact that such shoots have excellent potential to be exploited as a starting material for the development of axenic cultures in this herb species. The use of forced *A. aphyllus* shoots resulted not only in the establishment of axenic shoot cultures but also in their further successful maintenance, rooting and establishment, thus providing new information for micropropagation of *A. aphyllus*. Long term maintenance of such axenic shoot cultures is possible. The interactive role of auxins proves to be quite promising for rooting of regenerated shoots.

The method described for the first time in the present study provides an efficient reproducible protocol for *in vitro* propagation of *A. aphyllus* as a potent natural antitumor source. This protocol can also be used for the rapid

production of *A. aphyllus* plants, thus contributing to the germplasm conservation of this endangered and valuable medicinal species in the wild. The application of the protocol will facilitate research into the improved production of antitumor components via different biotechnological strategies, such as cell suspension, tissue and organ cultures, and large-scale cultivation in bioreactors. This study recommends that the obtained model in this study can be applied for extraction of bioactive compounds from different types of plants.

These results clearly demonstrate the potential of *in vitro* cultivation of this plant as a source of pharmaceutically important metabolites such as cytotoxic triterpenoid saponins. This could be of special interest for future biotechnological approaches.

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الإكثار المعملي وإنتاج المادة الفعالة من نبات الأسبرجس البري كنبات النادر

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ينمو نبات الأسبرجس طبيعيًا في مصر كنبات نادر ويستخدم في غذاء الإنسان ودواءه، ويعرف بالعربية باسم الهليون. ويستخدم في الطب البديل في علاج العديد من أمراض الكبد، والسرطان والملاريا. تم زراعة القمم النامية للنبات على بيئة موراشج وسكوج المضاف إليها تركيزات مختلفة من الكينتين والبنزيل أدنين ومع تركيزات مختلفة من نفثالين حامض الخليك لتكوين التضاعف العددي للأفرع. ثم نقلت على بيئة تجذير تحتوي على نصف قوة أملاح موراشج وسكوج مع تركيزات مختلفة من أندول حامض البيوتريك أو كل قوة أملاح بيئة موراشج وسكوج مع الكينتين وتركيزين من نفثالين حامض الخليك لتكوين الجذور. كانت أفضل النتائج في التضاعف على بيئة تحتوي على بنزيل أدنين بتركيز ١ مليجرام /لتر مع ٠.٢ مليجرام /لتر من نفثالين حامض الخليك وكان عدد الأفرع ١٢ فرع، وبطول ٦ سم مع تركيز ١ مليجرام /لتر بنزيل أدنين مع ٠.٤ مليجرام/لترمن نفثالين حامض الخليك. وكانت أعلى نسبة تجذير (٢٠٪) بعد ثلاثة أشهر من الزراعة على نصف قوة أملاح موراشج وسكوج مع أندول حامض البيوتريك بتركيز ١.٥ ،٢ مليجرام /لتر. وكان متوسط عدد الجذور المتكونة ١.٦ جذر، وبطول ٤ سم. وكان أعلى وزن للكالس الطازج ١٤.٠٣ جرام، عند استخدام ٢ مليجرام/ لتر نفثالين حامض الخليك مع ١ مليجرام/ لتر ٤،٢ ثنائي كلورو فينوكسي حامض الخليك مع ٠.٥ مليجرام/ لتر بنزيل أدنين حيث كان الكالس ذو قوام هش. وكانت نتائج التقدير للصابونين الكلي أعلى في النبات الأم (٥.٨٥ مليجرام / جرام) مقارنة بالكالس.