

In vitro Propagation of Ferns (*Asplenium nidus*) via Spores Culture

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ABSTRACT

As an alternative to seed propagation, an efficient micropropagation of *Asplenium nidus* and subsequent rooting were developed as an option for in vitro conservation purpose. Spores were used as an explant. Different growth hormones were used to study the response of fern to the different stages of propagation in vitro, consequently, to determine the best hormone concentration that give up best initiation and proliferation for prothalli, differentiation and rooting. Results show that all studied treatments increased the mean of prothalli length, width and height and the highest values (2.64 cm, 2.44 cm and 2.81 cm for prothalli length, width and height respectively) were observed when MS medium were supplemented with 3.0 mg/L KIN (kinetin) + 0.1 mg/L NAA (naphthalene acetic acid), meanwhile, the lowest values (0.84 cm, 0.59 cm and 0.96 cm for prothalli length, width and height respectively) were observed in control. A maximum of 5.28 shoot per microshoot were obtained on Murashige and Skoog agar medium supplemented with 2.0 mg/L 6-benzyl amino purine (BAP) and 0.1 mg/L of IBA (indole-3-butyric-acid). The highest leaves number (4.56) and stem height (5.50 cm) were observed when BAP and IBA were added respectively at 2.0 mg/L and 0.3 mg/L. Maximum root number (8.91 root/ex-plant) was obtained from media contained 1.0 mg/L naphthalene acetic acid (NAA). Meanwhile, maximum roots length (6.23 cm) and leaves number (5.39) were observed when NAA was added at 0.5 mg/L. *In vitro* propagation of fern can be applied to produce those species of ferns that are hard to propagate conventionally for the benefit of the ornamental industry.

Keywords: *Asplenium nidus*, Micropropagation, Spores, Root Formation, Shoot Formation.

INTRODUCTION

Ferns and club mosses belong to the Pteridophyta, and like Bryophytes have two distinct generations, a spore-phyte which bears asexual spores and a gametophyte which bears the sexual reproductive organs (Fernandez and Revilla, 2003).

Ferns are very ancient family of plants that are older than land animals and have worldwide distribution growing in all continents except Antarctica and most islands favoring moist temperate and tropical regions (Burkhill, 1935). Among those, *Asplenium nidus* commonly called Bird's Nest Fern belongs to the family Polypodiaceae. *A. nidus* is herbaceous, perennial in nature and native of tropical Asia. *A. nidus* is an epiphytic fern with a short vertical rhizome and large undivided leaves (Burkhill, 1935). Morphologically, it is a seedless, Vascular plant with erect, simple, wavy bright green leaves. Due to its unique shiny beautiful leaves it has attracted great importance in ornamental industry and in landscaping especially in the area where

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Received on 14/10/2012 and Accepted for Publication on 30/4/2013.

it is uncommon. These plants not only are soothing and add beauty but also can help in cleaning up our air naturally (Fernandez and Revilla, 2003). This fern is found abundantly in the rain forests where its germination and differentiation is quite easier due to favorable environmental conditions in that habitat. Its propagation under unfavorable climate is very difficult and slow (Bertrand *et al.*, 1999).

Ferns are often propagated by sowing spores onto sterilized soil. Propagation from spores can also be carried out in sterile culture. Spores must be collected from fronds bearing mature sporangia. Entire fronds can be left to dry above a sheet of paper, on which the spores are shed. Although it is said that spores of some species can be stored dry at low temperatures, their viability is usually highest when they have been freshly collected. Murashige and Skoog medium (1962) medium has been employed previously for the shoot culture of *Adiantum*, *Davallia*, *Nephrolepis* and *Playcerium*. The supplement of vitamins is unnecessary. Sugars are probably not required for spore's germination, but the growth of gametophytes is more rapid when they are present. A low concentration of glucose or sucrose (2.5-20 g/L) is usually improved it is propagation (Higuchi *et al.*, 1987). A study by Paek *et al.* (1984) showed that growth and multiplication of *Nephrolepis* was better with the combination of both kinds of growth regulator than with cytokinin alone. Although other varieties of *Nephrolepis exaltata* could be multiplied on media containing kinetin and NAA, for 'Scottii', 2-iP plus NAA was preferable (Paek *et al.*, 1984). Moreover, Higuchi *et al.* (1987) reported that *Nephrolepis cordifolia* cultured on 1/4 MS medium with 0.5 mg/LBAP were formed a high multiplication rate. On the other hand, Higuchi and Amaki (1989) reported that free hormone medium produce high multiplication rate.

Regeneration of plants from *Adiantum* and *Rumohra*

required the presence of 0.5-1 mg/L NAA. The rate of growth and proliferation of green globular bodies varied considerably between species. Sporophytes grown from fragmented tissues on media without growth regulators, form roots spontaneously and can be transferred soil for acclimatization without further treatment. The shoots obtained from 'shoot' culture on media containing high phosphate and a cytokinin can probably be rooted directly *ex vitro* in cool shaded conditions. However, Paek *et al.* (1984) reported that *in vitro* plant of *Nephrolepis exaltata* were rooting on 1/2 MS medium. This study demonstrated the first report of a simple reliable in clonal propagation of *in vitro* grown plantlets of *A. nidus* via spore culture.

MATERIALS AND METHODS

This work was conducted at Green Houses Tissue Culture Labs., Lattakia, as well as Plant Tissue Culture Laboratory, Faculty of Agriculture, Damascus University during the period from 2010-2011.

Plant Material:

A. nidus ferns of about three years old were growing in the nursery of Green Houses Labs., Lattakia, Syria, was utilized as mother plant material for obtaining the spores which used as an explants for *in vitro* micropropagation. *A. nidus* spores are morphologically brown in color, round and oval in shape found underneath the leaves of *A. nidus* (Fig.1).

Preparation and Sterilization of Spores Explants:

A. nidus leaves were carefully cut from mother plants growing in the nursery and taken immediately to the lab. The leaves were washed thoroughly under running tap water for 15-20 min., prior to sterilization procedures. The isolated spores were soaked under aseptic conditions in 70% ethanol for 10 sec. Then soaked in 20% Clorox

(5.25% Sodium hypochlorite NaOCl) for 15 min. Tween 20 (0.1%) was added as surfactant. The spores were then rinsed 3 times for 5 min each time in sterile de-ionized. All sterilization procedures were carried out in sterile environment under laminar Flow Cabinet.

Nutrient Medium:

The MS (Murashige and Skoog , 1962) basal medium was used in this study .

PH of the prepared media was adjusted at 5.7 ± 0.1 prior to addition agar. Medium was distributed into the culture Jars (325 ml) where each jar contained 45 ml of the medium. The Jars were immediately capped with polypropylene closer, and then it was autoclaved at 121°C (15 lbs/inch for 20 min).

The Initiation (Establishment) Stage:

The MS basal medium without growth regulators was used as establishment media for spore germination. 3% sucrose was added as carbon source. The PH (5.7 ± 0.1) was adjusted before adding agar as gelling agent (0.5 %). The spores were then scratched using scalpel and sprinkled over media. Spores germination was initiated. Spores were incubated at day and night temperature of $22 \pm 2^\circ\text{C}$ in dark for 12 weeks. Spores germinations were initiated under the mentioned conditions. Established explants resulted from the initial medium were used in the following multiplication stage.

The Multiplication Stage:

Germinated spores which formed prothalli were transferred on to multiplication MS basal media supplemented with 3% sucrose + 0.3% agar. Growth regulators were added as follows:

- 0.0 mg/L kin + 0.0 mg/L NAA
- 0.5 mg/L kin + 0.1 mg/L NAA
- 0.5 mg/L kin + 0.3 mg/L NAA

- 1.0 mg/L kin + 0.1 mg/L NAA
- 1.0 mg/L kin + 0.3 mg/L NAA
- 2.0 mg/L kin + 0.1 mg/L NAA
- 2.0 mg/L kin + 0.3 mg/L NAA
- 3.0 mg/L kin + 0.1 mg/L NAA
- 3.0 mg/L kin + 0.3 mg/L NAA

Each treatment consists of 3 replicates; each replicate consists of 3 jars, each containing 3 explants. Cultures were incubated in the growth chamber for four weeks at $24 \pm 2^\circ\text{C}$ light was provided by florescent lamps giving intensity of 2000 lux for 16 hours per day. The prothalli explants were repeatedly sub cultured two times at 30 days intervals into corresponding multiplication fresh medium. After two month data were as follow: average mean length height and width of prothalli.

The Differentiation Stage:

Vigorous prothalli explants were transferred individually to 1/2 MS basal media supplemented with 3% sucrose + 100 mg/L inositol + 0.4 mg/L thiamine + 255 mg/L sodium phosphate + agar at 0.5%. Growth regulators were supplemented as follows:

- 0.0 mg/L BAP + 0.0 mg/L IBA
- 1.0 mg/L BAP + 0.01 mg/L IBA
- 1.0 mg/L BAP + 0.05 mg/L IBA
- 2.0 mg/L BAP + 0.01 mg/L IBA
- 2.0 mg/L BAP + 0.05 mg/L IBA
- 3.0 mg/L BAP + 0.01 mg/L IBA
- 3.0 mg/L BAP + 0.05 mg/L IBA
- 4.0 mg/L BAP + 0.01 mg/L IBA
- 4.0 mg/L BAP + 0.05 mg/L IBA

Each treatment consists of 3 replicates; and each replicate consists of 3 jars, where each jar containing 3 prothalli culture explants. Cultures were incubated in growth chamber for 3 months at $24 \pm 2^\circ\text{C}$ light was provided by florescent lamps giving intensity of 1600 lux for 16 hours per day. At the end of this stage (3

months after culturing and differentiations media). The following data were recorded: multiplication rate, number of leaves and length of plantlets.

Rooting Stage:

Differentiated shoots were separated *in vitro* and transferred individually to rooting 1/2 MS media supplemented with 2% sucrose + agar at 0.3%. NAA was added in four concentrations (0, 0.5, 1, or 1.5 mg/L). Each treatment consists of 3 replicates, Each replicate consists of 3 plantlets. Cultures were incubated at 24 ± 2 °C, light was provided by florescent lamps giving intensity of 2000 lux for 16 hours per day. Four weeks later data were recorded on rooting rate, roots number, roots length, leaves number and shoots length). In the last phase of experiment, rooted plants were shifted to greenhouse before acclimatization. Various supporting media like garden sand, farm yard manure, and their combination were used.

Statistical Analysis:

Data were subjected to ANOVA analysis, and means were estimated. Mean values were compared according to least significant differences test (LSD) at 0.05 probability. Data were analyzed employing costat program (Duncan, 1979).

RESULTS AND DISCUSSION

1- Prothalli Initiation and Proliferation:

After two months from culturing on initiation and multiplication medium, it was observed that the initiated spores became swollen and a small green heart like structure appeared called Prothalli (Fig. 2). It was observed from data presented in Table (1) that all

studied treatments increased the mean of prothalli length, width and height and the highest values (2.64 cm, 2.81 cm and 2.81 cm for prothalli length, width and height respectively) were observed when medium were supplemented with 3.0 mg/L KIN + 0.1 mg/LNAA (Fig. 3). Lowest values (0.84 cm, 0.59 cm and 0.96 cm for prothalli length, width and height respectively) were observed in control. Kinetin at high concentration stimulate the good formation of prothalli compared with lower concentrations (0.5, 1.0 or 2.0 mg/L). This might be attributed to that cytokinins have been shown to activate RNA synthesis and to stimulate protein synthesis and enzyme activity (Kulaeva, 1980). Cytokinins might be required to regulate the synthesis of proteins involved in the formation and function of the mitotic spindle apparatus. However at low cytokinin concentration division of cell cycle is limiting. Subculture of the tissue onto a medium containing a cytokinin can then cause the cells to divide synchronously after a lag period (Jouanneau, 1975).

The supplement of auxin NAA (0.1 mg/L) promote initiation and multiplication of prothalli compared with higher concentration (0.3 mg/L). Schiavo *et al.* (1989) found that auxins cause DNA to become more methylated than usual and suggested that this might be necessary for the re-programming of differentiated cells. A low concentration of auxin is often used in conjunction with high levels of cytokinin at Stage II when shoot multiplication is required, although in some cases cytokinin alone is sufficient. It is important to choose an auxin at a concentration which will promote growth without inducing callus formation (Peaud-Lenoel and Jouanneau, 1980).

Table 1. Effect of kinetin in combination with NAA on proliferation of fern prothalli.

Growth regulator (mg/L)	Mean shoot length (cm)	Mean width (cm)	Mean height (cm)
0.0 KIN + 0.0 NAA	0.84 bc	0.59 c	0.96 d
0.5 KIN + 0.1 NAA	1.33 b	1.15 bc	1.54 bc
0.5 KIN + 0.3 NAA	0.93 b	0.68 c	1.06 d
1.0 KIN + 0.1 NAA	1.94 ab	1.84 b	2.06 b
1.0 KIN + 0.3 NAA	1.56 b	1.42 b	1.71 b
2.0 KIN+ 0.1 NAA	2.09 ab	2.11 a	2.54 a
2.0 KIN + 0.3 NAA	1.97 ab	1.85 b	2.06 b
3.0 KIN + 0.1 NAA	2.64 a	2.44 a	2.81 a
3.0 KIN + 0.3 NAA	2.32 a	2.31 a	2.62 a
LSD	0.61	0.49	0.37

Means followed the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$

2- Differentiation Stage:

The presences of BAP and IBA enhanced shoot development after three month weeks in culture (Table 2). Plant growth regulator free medium did not affect shoot proliferation. MS medium supplemented with 2.0 mg/L BAP and 0.1 mg/L IBA produced multiplication rate of 5.28 after three month growth periods. This was higher ($P=0.05$) than number of microshoots produced from MS medium supplemented with 4.0 BAP and 0.3 mg/L IBA. Moreover, regardless to the concentration of cytokinin BAP under study it was observed that low concentration of auxin IBA (0.1) increased the multiplication rate comparing with high concentration of auxin (0.3). This because cytokinin promote cell division and multiplication rate in plants (Pyott and Joverse, 1981). Specially when used in low concentrations or when cultured explants are very small in size that has high response for cell division (Huang, 1984).

Medium supplemented with auxin at two concentrations in combination with cytokinin promote the growth and formation of new shoots, consequently, increasing

multiplication rate (Pierik, 1987). This depend on plant specie, physiological state and age of mother plant, explant type, source, type and concentration of used hormones (Conti *et al.*, 1991). At two auxin concentration multiplication and differentiation stages increasing permeability rate of plant cells, osmosis, modifying the osmotic system, increasing the rate of transcription of genetic material and formation of new proteins (Rossignol *et al.*, 1990). Moreover, high concentrations of auxin (0.3 mg/L) increased rate of stem elongation compared with low concentration (0.1 mg/L) and maximum shoot length was 5.50 cm. Moreover, using of cytokinin in lower concentration (1.0 mg/L) increased stem elongation comparing with higher concentrations (3.0 and 4.0 mg/L). This might be attributed to that cytokinin in hormone of cell division rather than cell elongation. Addition of cytokinin and auxin to MS medium produced few leaf number after three month of culture. However, 3.0 mg/L BAP and 0.3 mg/L IBA produced an average number of leaves of 4.56 shoots per explants as compared with the control (1.69 shoots per explants). Maximum shoot

length was 5.5 cm at 3.0 mg/L BAP and 0.3 mg/L IBA and it did not have any statistical significant compared with 2.0

mg/L BAP and 0.1 mg/L IBA.

Table 2. Effect of BAP in combination with IBA on differentiation of fern.

Grwoth regulators (mg/L)	Multiplication rate	Leaves number	Stem height (cm)
0.0 BAP + 0.0 IBA	1.38 f	1.69 d	1.40 d
1.0 BAP + 0.1 IBA	1.75 ef	2.76 d	2.70 c
1.0BAP + 0.3 IBA	1.28 f	2.77 d	2.66 c
2.0 BAP + 0.1 IBA	5.28 a	4.42 a	4.90 a
2.0 BAP + 0.3 IBA	3.78 bc	4.56 a	5.50 a
3.0 BAP + 0.1 IBA	4.18 b	3.84 a	3.76 b
3.0 BAP + 0.3 IBA	2.35 de	4.33 a	4.75 a
4.0 BAP + 0.1 IBA	3.52 c	2.93 bc	2.93 c
4.0 BAP + 0.3 IBA	2.62 d	3.66 ab	3.93 b
L.S.D	0.62	0.87	0.73

Means followed the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$.

Rooting stage

Rooting is one of the most difficult stages to be resolved in plants using tissue culture. In this study, the results showed that shoots were successfully rooted on MS medium supplemented with different concentrations of auxins (Tables 3). Rooting percentages, as well as the number of roots per shoot, were evaluated after four weeks.

Root initiation was achieved from the bases of shoots, after incubation on media containing NAA in various concentrations. There were significant differences in the percentage of shoots developing roots. Maximum root number was obtained with the addition of 1.0 mg/L NAA, with an average of (5.3 mm) root length compared with the control (3.52 mm). Rooting percentage increased up to 93% with 1.0 mg/L NAA. Root length was significantly affected with the use of NAA compared with the control (Table 3).

Concerning leaves number, the lowest leaves number (3.62) was observed in control and the highest leaves number (5.39) was observed when NAA was added at 0.5 mg/L, followed with no significant differences by treatment with 1.0 mg/L NAA. Auxin action on roots level is similar to its action on stem level but, roots are more susceptible so low concentration of auxin promote and stimulate rooting and increase rooting rate on the other hand, high concentration of auxin inhibit rooting, decrease roots elongation and causes induction of callus tissue (Gupta, 1986; Fitchet, 1987 and Vuylsteke, 1989). Physiologically, auxin has important role on RNA metabolism, proteins synthesis via transcription of neur mRNA. It has indirect role on including enzyme activity of ATPase in the cell walls, this enzymes are responsible of ions transport (OH^- , H^+). In addition, auxin increasing rate of cell permeability for protons and other ions, consequently, ions exchange

(Rossignol *et al.*, 1990). Auxin might not have stimulatory effect on cell rooting stages but it has direct and key role on

roots induction (Haissing, 1986) and cell elongation (Rossignol *et al.*, 1990).

Table 3. Effect of NAA on rooting of fern plantlets.

Concentration (mg/L)	Rooting rate (%)	Roots number	Roots length (cm)	Leaves number	Stem length (cm)
0.0	69.30 c	4.78 d	2.25 d	3.62 c	4.75 b
0.5	70.23 c	6.18 c	6.23 a	5.39 a	4.50 b
1.0	93.33 a	8.91 a	5.30 b	5.02 ab	6.75 a
1.5	88.03 b	8.18 b	3.60 c	4.42 b	6.90 a
L.S.D	3.68	0.46	0.17	0.61	0.73

Means followed the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$



Fig (1-6): In vitro propagation of *Asplenium nidus* fern.

(Fig. 1) Spores underneath *Asplenium* leaf. (Fig. 2) Prothalli initiation *in vitro* via spore (Fig. 3) Prothalli multiplication. (Fig. 4) Differentiation. (Fig. 5) Rooting. (Fig. 6) Acclimatization.

CONCLUSION

In vitro propagation of fern via spore culture was achieved. The best prothalli initiation and proliferation was observed when spores were cultured on MS agar medium supplemented with 3.0 mg/L KIN + 0.1 mg/L NAA. The best shoots number was observed when BAP and IBA were added respectively at 2.0 mg/L and 0.1

meanwhile, the best leaves number and stem height were observed when BAP and IBA were added respectively at 2.0 mg/L and 0.3 mg/L. NAA at 1.0 mg/L resulted in the best rooting rate and roots number, meanwhile, the highest root length and leaves number were observed when NAA was added at 0.5 mg/L.

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الإكثار الخضري الدقيق للسرخس (*Asplenium nidus*) عن طريق زراعة الأبواغ مخبرياً

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ملخص

كبدل عن الإكثار البذري، تم تطوير تقنية لإكثار سرخس الأسبلينيوم (*Asplenium nidus*) و تجذيره و حفظه مخبرياً. استخدمت الأبواغ خزعات نباتية. واستخدمت هرمونات نمو مختلفة لمعرفة استجابة نبات السرخس لمراحل الإكثار المختلفة في المخبر، ومن ثم تحديد أفضل التراكيز الهرمونية التي تساعد في تكون الثالوس الأولي وإكثاره ثم تكشفه وتجذيره. أظهرت النتائج أن المعاملات المدروسة جميعها قد زادت من متوسط طول، وعرض، وارتفاع كتلة الثالوس الأولي المتكونة، وقد لوحظت أعلى القيم (2,46 سم، 2,44 سم، 2,81 سم بالنسبة لطول، وعرض، وارتفاع كتلة الثالوس على التوالي) عند الزراعة على بيئة مضاف إليها 3 مغ/ل كينتين + 0.1 مغ/ل نفتالين حمض الخليك، بينما لوحظت أقل القيم (0.84 سم، 0.59 سم و 0.96 سم بالنسبة لطول، وعرض، وارتفاع الثالوس على التوالي) في نباتات الشاهد. ولوحظ أكبر عدد للأفرع (5.28) عند الزراعة على بيئة موراشيج وسكوغ المهلمة بالأجار المضاف إليها 2مغ/ل بنزاييل أمينو بيورين + 0.1 مغ/ل إندول حمض البيوتريك. أما أكبر عدد للأوراق (4.56) و أعلى طول للساق (5.5)، فقد لوحظت عند إضافة بنزاييل أمينو بيورين و إندول حمض البيوتريك بتركيز 2.0 مغ/ل و 0.3 مغ/ل على الترتيب. أدى استخدام نفتالين حمض الخليك بتركيز 1.0 مغ/ل إلى أكبر عدد الجذور (8.91) بالمقارنة مع التراكيز الأخرى المستخدمة، بينما لوحظ أعلى طول للجذور (6.23 سم) و أكبر عدد للأوراق (5.39) عند إضافة نفتالين حمض الخليك بتركيز 0.5 مغ/ل. يمكن استخدام تقنية الإكثار الخضري الدقيق في إكثار أنواع السرخسيات التي يصعب إكثارها بالطرق التقليدية؛ لما لها من أهمية تزيينية اقتصادية .

الكلمات الدالة: سرخس أسبلينيوم، إكثار خضري دقيق، أبواغ، تكون جذور، تكون أفرع.

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تاريخ استلام البحث 2012/10/14 وتاريخ قبوله 2013/4/30.