Pak. J. Bot., 40(1): 91-97, 2008.

IN VITRO PROPAGATION OF BIRD'S NEST FERN (*ASPLENIUM NIDUS)* FROM SPORES

SAIFULLAH KHAN, MARIAM RAZIQ AND HAMMAD AFZAL KAYANI

Plant Tissue Culture and Biotechnology Division, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.
E-mail:drsaif@super.net.pk, Phone: (92-21) 4819017

Abstract

Micropropagation can be applied to produce those species of fern that are hard to propagate conventionally for the benefit of the ornamental industry. The purpose of this study was to assess the behavior of the *Asplenium nidus* under *in-vitro* conditions. Micropropagation of *Asplenium nidus* was initiated using spores as an explant. A series of experiments were conducted to evaluate best sterilization technique for *Asplenium nidus* spores. Commercial bleach at 20% (v/v) along with few drops of Tween-20 was found very effective. Explants were cultured on half-strength Murashige & Skoog (MS) basal medium and were incubated in the dark at 22±2°C. After 12 weeks germination of spores, prothalli were obtained. After germination, cultures were transferred on to fresh medium every 4-week. Prothalli were subcultured on half-MS medium along with Benzylaminopurine (BAP) (1-4 mgl^{-1}) and Naphthelenacetic acid (NAA) (0.1-0.5 mgl^{-1}) where they multiplied successfully. Maximum number of shoots was obtained after transferring explant on differentiation media containing various concentration of Sodium dihydrogen phosphate (NaH_2PO_4) along with NAA and BAP at the concentration of 2 mgl^{-1} and 0.5mgl^{-1} each, respectively. Addition of Sodium dihydrogen phosphate in MS medium plays a significant role in differentiation from gametophyte stage to sporophytic stage. Rooting was optimal at concentration of 2.0 mgl^{-1} Indole Butyric acid (IBA). Best material for acclimatization was found to be garden sand.

Introduction

Ferns are very ancient family of plants that are older than land animals. Ferns 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*. It is herbaceous; perennial in nature and native of tropical Asia. *Asplenium 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 it is uncommon. These plants not only are soothing and add beauty but also can help in cleaning up our air naturally (Fernandez & Revilla, 2003).

Fern also have medicinal uses, a decoction or infusion of the plant is taken by the Sakai of north Pahang to ease labor pains. The plant is also used as a depurative. Malays used the lotion made from the leaves to reduce fever and as a hair wash (Burkhill, 1935).

Asplenium nidus like other ferns passes through two distinct phases during their lifecycle, the small, simple haploid gamete producing phase, gametophyte and the large morphologically complex, diploid spore producing phase, sporophyte (Fernandez & 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 in response. Its spore germination and conversion of gametophytic stage (haploid) into sporophytic stage (diploid) is easily understandable under *In vitro* conditions (Bertrand *et al*., 1999, Fernandez & Revilla, 2003).

This study was conducted to develop the optimized protocol for production of *Asplenium nidus* as an ornamental and commercial commodity on mass scale. Moreover, the work is under progress to exploit its medicinal and pharmacological aspects.

Material and Methods

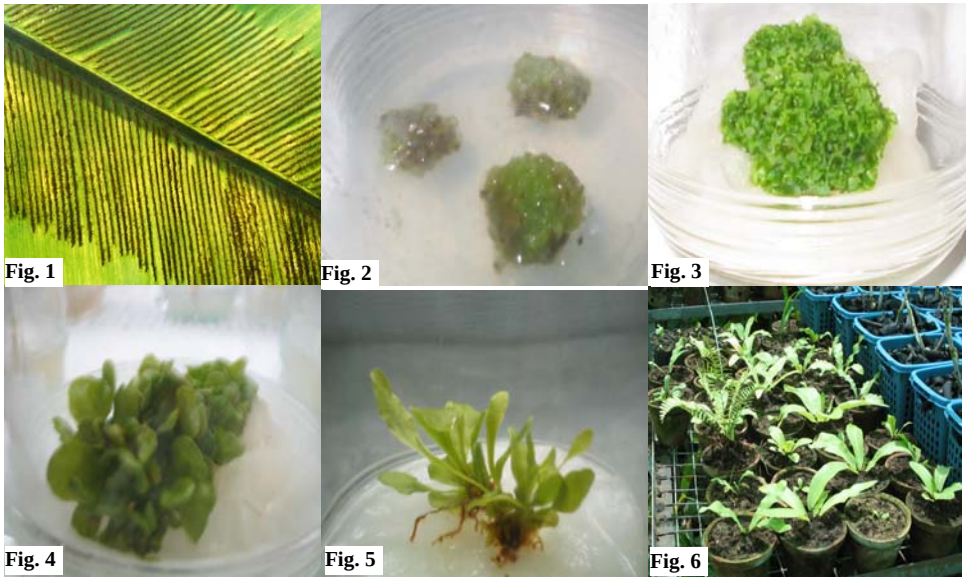
Spores were used as an explant in this study for *In vitro* propagation and were collected from the nursery of International Center for Chemical & Biological Sciences (ICCBS), University of Karachi. *Asplenium nidus* spores are morphologically brown in color, round and oval in shape found underneath the leaves of *Asplenium nidus* (Fig. 1) (Fernandez & Revilla, 2003). Spores were collected from a single plant that was three years old. The leaves were washed thoroughly under running tap water for 15-20 min., prior to sterilization procedure.

For spores sterilization, different surface sterilants like Sodium hypochlorite, Calcium hypochlorite and Mercuric chloride were used along with 1-2 drops of tween-20 as a wetting agent in 200ml distilled water for different exposure time. The spores were sterilized by three rinses in sterile distilled water for 5 minutes each to remove the traces of sterilant, and soaked in filter paper. All the sterilization work was carried out in sterile environment under Laminar flow cabinet (Bertrand *et al*., 1999).

Half-strength MS medium (Murashige & Skoog, 1962) without growth regulator were used as medium for germination of spores, 2% sucrose was added as a carbon source, the pH 5.75 was adjusted using 1N HCl and 1N NaOH as required. Phytagel was used as a solidifying agent at a concentration of 0.25% w/v. The spores were scratched using scalpel and sprinkled over media. Spores germination was initiated and media were incubated at 22±2 ºC in dark for 12 weeks.

After germination, spores (now prothalli) were transferred on to multiplication media containing half-strength MS medium (Murashige & Skoog, 1962) with growth regulators BAP (1-4 mgl^{-1}) and NAA (0.1-0.5 mgl^{-1}) each respectively with 3% sucrose and 0.25% w/v phytagel (Table 2). Cultures were incubated in the growth room for four week at 24±2°C, at 2000 lux for 16h photoperiod. There were at least five replicates per formulation. Data were recorded every week for average mean length and height of plants (prothalli).

Plants multiplying vigorously were transferred on to differentiation media. Five different types of media were used for *In vitro* differentiation of fern as described in Table 3 (Murashige & Skoog, 1962). All five media corresponds half-strength MS media with varying concentrations of NaH_2PO_4, containing growth regulators BAP and NAA with the concentration of $3mgl^{-1}$ and $0.5mgl^{-1}$, 3% sucrose was added in all combinations at pH 5.75, and 0.25 w/v phytagel was added as a supporting material. Cultures were incubated in the growth chamber for four weeks. A layer of liquid media was suspended above the solid media to facilitate the fertilization process. Data was recorded every week for average number of plants, number of leaves, average length, height and width of plants. Differentiated plants were then transferred on solid medium for root formation, in this study half-strength MS media containing NaH_2PO_4, with different concentration (0-$2.5mgl^{-1}$) of IBA was used.



Micropropagation of *Asplenium nidus*.
1. Spores underneath *Asplenium* leaf, 2. Prothalli formation in-vitro from spores, 3. Multiplication, 4. Differentiation, 5. Rooting, 6. Acclimatization

All experiments were repeated at least twice and completely randomized design was used in the experiment and the data subjected to square root transformation prior to analysis. Data were analyzed employing Minitab version (11.12).

In the last phase of experiment, rooted plants were shifted to greenhouse for acclimatization. Various supporting media like garden sand, farm yard manure, charcoal and combination of all three were used and data of this study along with some other experiments will be published later.

Result and Discussion

In vitro propagation of *Asplenium nidus* was investigated during this study and for this purpose, a series of experiments were performed to optimize conditions for multiplication of *Asplenium nidus* plants (Bertrand *et al*., 1999).

Effect of sterilants: Three different surface sterilants were used for sterilization of spores. As spores are sensitive and the mortality rate of spores is high, so 20% (v/v) commercial bleach along with 2 drops of tween-20 was found to be best for sterilization of spores, as below 20% the spores were not sterile and above 20%, high mortality was observed due to increase in permeability of Sodium hypochlorite inside the cells of spores. Sterilization using Calcium hypochlorite showed little response as the explant initiated got contamination, and Mercuric chloride sterilization showed although better response but not as good as sodium hypochlorite which is also less toxic to environment and the worker (Table 1). This can be deduced that the concentration used and length of exposure varies greatly depending on the type of plant material (Banks, 1999). The use of tween-20 ensures the contact of sterilant with the explant thoroughly by reducing surface tension and removing air bubbles.

Table 1. Different Sterilants used for Spore sterilization.

Sterilant	Treatments	% Conc. (v/v)	Time (min.)
Control (w/o sterilant)	T0	0	10
Commercial bleach	T1	15	10
(0.5% Sodium hypochlorite)	T2	20	10
	T3	25	10
Calcium hypochlorite	T4	15	10
	T5	20	10
	T6	25	10
Mercuric chloride	T7	0.01	5
	T8	0.02	5
	T9	0.03	5

Table 2. Effect of BAP in combination with NAA on *in-vitro* multiplication of fern.

Media Codes	BAP (mgl^{-1})	NAA (mgl^{-1})	Mean length + SE (cm)	Mean width + SE (cm)	Mean height + SE (cm)
F1	0	0	0.23 ± 0.063	0.27 ± 0.029	0.30 ± 0.026
F2	1	0.1	0.47 ± 0.030	0.42 ± 0.024	0.43 ± 0.053
F3	2	0.1	0.53 ± 0.041	0.45 ± 0.023	0.52 ± 0.038
F4	3	0.1	0.56 ± 0.020	0.51 ± 0.041	0.56 ± 0.030
F5	4	0.1	0.57 ± 0.026	0.55 ± 0.095	0.57 ± 0.023
F6	1	0.5	0.59 ± 0.028	0.60 ± 0.054	0.61 ± 0.018
F7	2	0.5	0.80 ± 0.011	0.82 ± 0.039	0.83 ± 0.007
F8	3	0.5	0.71 ± 0.041	0.70 ± 0.033	0.73 ± 0.008
F9	4	0.5	0.63 ± 0.036	0.63 ± 0.026	0.66 ± 0.017

SE = Standard Error

Table 3. Effect of NaH_2PO_4 on *In vitro* differentiation of *Asplenium* plant.

Media codes	NaH_2PO_4 (mgl^{-1})	Mean no. plants ± SE (cm)	Mean no. leaves ± SE (cm)	Mean length ± SE (cm)	Mean width ± SE (cm)	Mean height ± SE (cm)
MF0	0	2.065 ± 0.200	2.255 ± 0.122	1.516 ± 0.023	1.568 ± 0.017	1.341 ± 0.026
MF1	150	2.296 ± 0.180	2.736 ± 0.129	1.549 ± 0.022	1.661 ± 0.016	1.529 ± 0.029
MF2	175	2.337 ± 0.214	2.878 ± 0.144	1.561 ± 0.028	1.612 ± 0.021	1.476 ± 0.028
MF3	200	2.625 ± 0.103	3.047 ± 0.136	1.618 ± 0.051	1.720 ± 0.015	1.617 ± 0.082
MF4	225	2.201 ± 0.256	2.946 ± 0.143	1.555 ± 0.027	1.655 ± 0.016	1.449 ± 0.024

SE = Standard Error

Table 4. Effect of IBA on *In vitro* rooting of *Asplenium nidus*.

Media codes	IBA Conc. (mgl^{-1})	Root emergence (days)	No. of roots	Root length ± SE (cm)	Field survival %
FI1	0.5	25-28	1	0.64 ± 0.034	<70
FI2	1.0	24-26	2	0.87 ± 0.013	<80
FI3	1.5	20-22	4	1.08 ± 0.006	80
FI4	2.0	18-21	5	2.31 ± 0.029	<90
FI5	2.5	13-16	5	2.57 ± 0.060	<70

SE = Standard Error

Spores have been used as the explant source for successful high frequency regeneration of plants. Although the regeneration of plants from spores is quite difficult *In vitro*, but optimization of every step from initiation to acclimatization makes it more feasible to produce plants from spores *In vitro* (Banks, 1999).

Prothalli formation: After four weeks, it was observed that the initiated spores on basal induction media containing simple half-strength MS medium became swollen and a small green heart like structure appeared called prothalli which are of 1cm in size as shown in Fig. 2 (Murashige & Skoog, 1962). Half-strength MS salt medium was used as experimental media during this study and has been cited previously for other ferns as well (Fernandez *et al*., 1997, Kyte & Kleyn, 1996). Regenerated prothalli of same size were subcultured on multiplication media (Table 2). Density of spores did not make any remarkable difference.

Multiplication: In this series of experiments, three different types of parameters were evaluated on 9 different media formulations. A profound effect was observed on the multiplication of plant in F7 (Table 2) media containing growth regulators BAP and NAA at the concentration of $2mgl^{-1}$ and $0.5mgl^{-1}$ respectively showed the significant increase in multiplication of plant (Fig. 1), where as low concentrations of BAP and NAA showed less number of plantlets and at higher concentration, explant started to show browning which confers the detrimental effect of the dose (Higuchi & Amaki, 1989). This reflects that plants need both auxins and cytokinins which should be supplied in the medium and the ratio between auxin and cytokinin seems to be very important for multiplication (Bertrand *et al*., 1999, Fernandez *et al*., 1999). There are many reports in which application of growth regulators are indicated that enhanced and suppress the plant growth which directly effect on rate of multiplication (Fernandez & Revilla, 2003).

It was observed that three to four fold increase subsequently occur in multiplication media when the plants subcultured onto fresh medium, this leads to produce large number of plant therefore, cyclic production of differentiated plant was possible ((Fernandez *et al*., 1999, Fernandez & Revilla, 2003).

Effect of Sodium dihydrogen phosphate on differentiation: After multiplication, differentiation stage was optimized. For this purpose, various experiments were conducted to establish the differentiation protocol as described in Table 3. After a period of four weeks incubation, axillary shoots started emerging from the green mass of cells. Abundant differentiation of leaves were observed on the differentiation media MF1, MF2, MF3 and MF4 containing various amount of NaH_2PO_4 along with growth regulator BAP and NAA with the concentration of 2 mgl^{-1} and $0.5mgl^{-1}$ respectively (Higuchi & Amaki, 1989). All media containing NaH_2PO_4 showed different degrees of differentiation (Table 3). An increase in axillary shoots was also observed upto a certain concentration of NaH_2PO_4 upto $200mgl^{-1}$ at its maximum while reduced growth was observed above the concentration 200 mgl^{-1} (Table 3). This reflects that NaH_2PO_4 plays a significant role in the differentiation process of *Asplenium nidus* (Kyte & Kleyn, 1996).

Although, Sodium dihydrogen phosphate (NaH_2PO_4) is routinely included in tissue culture media and it affects on plant maturation and growth (Kyte & Kleyn 1996) but its actual mechanism in the differentiation is still undefined. As cited previously, elevated levels of NaH_2PO_4 increased the differentiation of shoot buds (Sharma & Thorpe, 1989). Thus, it was concluded that the NaH_2PO_4 amount is directly related to *in vitro* differentiation of *Asplenium nidus* upto a certain optimum level.

Rooting: For root induction, experiments were also performed in order to optimize the rooting medium in which different auxins (IBA, NAA, and IAA) were used (Data not shown). The plants having multiple shoots were rooted easily on solid medium

containing IBA 2.0 mgl^{-1} (De Klerk *et al*., 1997). Control showed browning in differentiated leaves. Above this concentration, the plantlets started to show browning and also survival rate decreased. The plantlets having roots were then transferred to greenhouse for acclimatization (Fig. 5).

Acclimatization: *Asplenium nidus* needs a bright, warm, moist, humid condition with a well-drained supporting medium. For this purpose, experiments were conducted for efficient and economical acclimatization procedure under green-net conditions (Data not shown). The maximum survival of plants upto 95% were achieved on simple garden sand with occasional spraying of micronutrients. As a result, many plants have been successfully acclimatized and are growing vigorously having apple green shiny fronds (Fig. 6) (Bertrand *et al*., 1999).

A micropropagation protocol for initiation, multiplication and differentiation of *Asplenium nidus* is successfully optimized in which spores are used as an explant. Sterilization protocol was optimized by using different surface sterilants. Sterilized spores were initiated on basal induction media, spores regenerated and prothalli formed. These prothalli were subcultured on multiplication media for mass propagation, after this stage the plantlet were subcultured on differentiation media where axillary shooting increased and plant started differentiation. Thus, from a single explant it is possible to regenerate hundreds of plants by using micropropagation technique. It is the fastest and economical method for the propagation plants that are hard to propagate vegetatively. Although *In vitro* propagation of this exotic plant may have been done, but this study has some modifications as compared to previous work, and also conditions are optimized according to environment in which work executed. Future studies regarding biotransformation using *Asplenium* will be evaluated to check its bioactivity spectrum.

Acknowledgements

The authors would like to thank H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS) for providing the facilities in terms of equipments, chemicals and lab.

References

Banks, J.A. 1999. Gametophyte development in Ferns. *Annual Rev. Plant Physiology*, 50: 163-86.

Bertrand, A.M., M.A. Albuerne, H. Fernandez, A. Gonzalez and R. Sanchez-Tames. 1999. *In vitro* organogenesis of *Polypodium cambricum. Plant Cell Tissue & Organ Culture*, 57: 65-69.

Burkhill, I.H. 1935. *A Dictionary of the Economic Products of Malay Peninsular*. Ministry of Agriculture Malaysia, 1542.

De Klerk, G-J. J. Ter Brugge and S. Marinova. 1997. Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in Malus 'Jork 9'. *Plant Cell Tissue & Organ Culture*, 49: 39-44.

Fernandez, H., A.M. Bertrand and R. Sanchez-Tames. 1997. Plantlet regeneration in *Asplenium nidus* L. and *Pteris ensiformis* L., by homogenization of BA treated rhizomes. *Scien. Hort*., 68: 243-247.

Fernandez, H., A.M. Bertrand and R. Sanchez-Tames. 1999. Biological and nutritional aspects involved in fern multiplication. *Plant Cell Tissue & Organ Culture*, 56: 211-214.

Fernandez, H. and M.A. Revilla. 2003. *In-vitro* culture of ornamental ferns. *Plant Cell Tissue & Organ Culture*, 73: 1-13.

Higuchi, H. and W. Amaki. 1989. Effect of 6-benzylaminopurine on the organogenesis of *Asplenium nidus* L., through *In vitro* propagation. *Scien. Hort*., 37: 351-359.

Kyte, L. and J. Kleyn. 1996. *Plants from test tubes, An introduction to micropropagation*. Portland, OR: Timber Press; 240.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology Plant*, 15: 473-497.

Sharma, K.K. and T.A. Thorpe. 1989. *In-vitro* regeneration of shoot buds and plantlets from seedling root segments of *Brassica napus* L. *Plant Cell Tissue & Organ Culture*, 18-1: 129-141.

(Received for publication 20 April 2007)