EFFECT OF NAPHTHALENEACETIC ACID (NAA) ON THE IN-VITRO REGENERATION OF CHLOROPHYTUM

Aditi S. Pandey*
Assistant Professor, Department of Applied Chemistry
Priyadarshini Institute of Engineering and Technology, Nagpur
Email-Id: aditipandey2016@gmail.com

A B S T R A C T

The biotechnological research is multidisciplinary and acquires expertise of different areas. The techniques of plant tissue culture offers means for mass multiplication, for biomass energy production as well as for the conservation of important, elite and rare species that are threatened in nature with danger of extinction. Attempts have been made to standardize the different factors for in-vitro regeneration of some of the medicinal plants.Safed Musli is a medicinal plant used to overcome weakness. It is a well-known ayurvedic medicine rich in alkaloids, vitamins, minerals, protein, carbohydrate, steroids and polysaccharides.In this experiment Leaf segment, root segment and stem disc of two different collections Chlorophytum were collected from mature plants as explants from the field and brought to the laboratory.Different concentrations and combinations of NAA used for multiple shoot initiation and multiplication. Among all the concentrations and combinations tested in the present study, MS media with 1.0 mg/l NAA+ 0.5 mg/l KIN was found good for shoot initiation and multiplication.

Keywords: Chlorophytum , NAA, Tissue culture, Shoot initiation and multiplication

1. Introduction

Medicinal plants comprise a group of large number of plant species that produce raw material for pharmaceuticals and phyto-chemicals for manufacturing drugs. In the commercial market, medicinal herbs are used as raw drugs, extracts or tinctures. The World Health Organization (WHO) estimates that up to 80% of the world population rely on plants for their primary health care. The international medicinal plants market is worth US $60 billion per year, and growing at the rate of 7% per annum (Bhojvaid, 2003). Plants have contributed more than 7,000 different compounds in use as heart drugs, laxatives, diuretics, antibiotics, decongestants, analgesics, anesthetics, ulcer treatments anti-parasitic compounds and so on (Ved *et al.,* 1998).

A complete understanding of medicinal plants involves a number of disciplines including commerce, botany, horticulture, chemistry, enzymology, genetics, quality control and pharmacology. The number of medicinal plants in India, both indigenous and introduced, has been variously put at between 3,000 to 3,500 species of higher plants (Asolkar *et. al.* 1992; Chopra *et. al*., 1956, 1974). Sixteen medicinal plants of exotic origin, introduced in India from time to time, are under cultivation and are now considered a part of the Indian medicinal plant resources (Sarin, 2003).
A medicinal herb can be compared with a chemical factory due to presence of number of chemical constituents like alkaloids, glycosides, saponins, resins, oleoresins, sesquiterpene lactones and oils (essential and fixed). With introduction of sophisticated techniques, the scientists started exploring the plant flora for active constituents.

The biotechnological research is multidisciplinary and acquires expertise of different areas. Research consists of characterization and testing of different proveniences and genotypes, development of suitable multiplication methods for plants, plant cells or tissues, either in the field, in micro propagation conditions or in bioreactors. The techniques of plant tissue culture offers means for mass multiplication, for biomass energy

production as well as for the conservation of important, elite and rare species that are threatened in nature with danger of extinction (Jang *et al.,* 2003). Plant tissue culture techniques are now being used also for monitoring of their secondary metabolites. The production of secondary metabolites by tissue culture has commercial potential as well as being useful in studying the biosynthesis and regulation of secondary products. The potential of plant tissue culture both source of high value chemicals and as a system for studying secondary metabolism has not yet been exploited (Holden *et al*., 1987). *Curcuma* (Haldi) and *Chlorophtum* (Safed musli) are two endangered and rare medicinal plants of this state have been selected for *in-vitro* regeneration.

Safed Musli (*Chlorophytum*) is a medicinal plant that grows in dense forests. It belongs to Liliaceae, a family of 175 species distributed all over the world. Among the 13 species found in India, Safed Musli (*Chlorophytum biovirilianum*) has market potential (Borodia *et al*., 1995). Safed Musli is a medicinal plant used to overcome general and sexual weakness. It is a well-known ayurvedic medicine rich in alkaloids, vitamins, minerals, protein, carbohydrate, steroids and polysaccharides. Dried roots of *Chlorophytum* contain 42% carbohydrate, 8–9% protein, 3–4% fiber and 2–17% saponin (Borodia *et al*., 1995). Research studies on *Chlorophytum* conducted in India and elsewhere indicate that saponins are responsible for medicinal properties (Arora *et al*., 1999). The plant yields a flavonone glycoside, which is a powerful uterine stimulant. Mainly, Saponins are natural surfactants, or detergents, found in many plants.

With the increasing population, coupled with the shrinking of genetic diversity in traditional farming systems and reduction in the area of prime land available for agri-horticultural crops, there is emergent need for better utilization of plant genetic resources including lesser known or underutilized plant species like *Chlorophytum* through critical characterization and evaluation of existing biodiversity.Arora *et al*., (1999) developed an improved method for large-scale rapid multiplication of *C. borivillianum* through somatic embryogenesis. Somatic embryos were obtained on MS medium containing 2.25 micro molar 2,4-D and 1.5 micro molar kinetin. The technique developed is highly efficient to get miniature plantlets for field transfer in 2 months, starting from callus.

Purohit *et al*., (1994) reported *in-vitro* regenerated protocol for shoot multiplication of safed musli. Multiplication has been achieved on MS medium supplemented with 22.2 micro molar benzyladenine using young shoot bases as explant 67% of the micropropogated plants were successfully established in pots.

- Suri *et al*.,(1999) developeda method for rapid multiplication of *Curculigo orchioides* (Hypoxidaceae) through direct organogenesis and bulbil formation *in vitro*. Leaf and underground stem explants produced maximum number of shoots (four and 10 per explant, respectively) on B5 medium supplemented with 4.4 mM BAP. Higher concentrations of BAP (22 mM) in the medium completely inhibited the growth and shoot proliferation from the leaf explants. Stem-disc-derived callus produced numerous bulbils on the medium containing BAP (8.8±35.2 mM) and sucrose (0.12±0.23 M).

Materials & Methods

1. Standardization of *in- vitro* regeneration protocol

1.1 Selection of explants

Leaf segment, root segment and stem disc of *Chlorophytum* were collected from mature plants as explants from the field and brought to the laboratory.

1.2 Sterilization of explants

The source tissues were kept under running tap water (5 min) and washed with a 5 % laboline detergent for 5-10 mints. Explants (leaf- 1cm., root segment 1 cm, stem disc) from the fields were brought to laboratory and thoroughly washed in 2 per cent labolene solution to clear the dirt particles.

After dipping the explants in required sterilants, as specified in Table 1 for stipulated time period, these were washed at least four times with sterilized double distilled water before inoculation.

Surface sterilization treatments			
	Sterilants	**Steriliant Conc. (%)**	**Duration of treatment**
I	Labolene	2.0	5 min.
	Sod. Hypochlorite	2.0	10 min.
	$HgCl_2$	0.2	10 min.
	Ethanol	70	1 min
II	Labolene	2.0	5 min.
	Sod. Hypochlorite	2.0	15 min.
	$HgCl_2$	0.2	20 min.
	Ethanol	70	2 min
III	Labolene	2.0	5 min.
	Bavistin	1.0	15 min
	Sod. Hypochlorite	2.0	15 min.
	$HgCl_2$	0.2	20 min.
	Ethanol	70	2 min.
IV	Labolene	2.0	5 min.
	Bavistin	1.0	20 min
	Sod. Hypochlorite	2.0	20 min.
	$HgCl_2$	0.2	20 min.
	Ethanol	70	2 min.
V	Labolene	2.0	5 min.
	Bleaching Powder	1.0	15 min.
	Bavistin	1.0	20 min
	$HgCl_2$+Tween 20 (2 drop)	0.2	15 min.
	Ethanol	70	2 min.
VI	Labolene	2.0	5 min.
	Bleaching Powder+ ampicilline	1.0	20 min
	Bavistin	2.0	15 min.
	$HgCl_2$+Tween 20 (2 drop)	0.2	15 min.
	Ethanol	70	2 min.

Table 1 Surface sterilization treatments for *Chlorophytum*.

1.3 Media for shoot initiation

The materials were cut into appropriate sizes (leaf- 1cm., root segment 1 cm, stem disc) and cultured on MS medium (Murashige and Skoog, 1962). The medium was used at different strengths. The pH of the medium was adjusted to 5.8 before autoclaving at a pressure of 15 lbs for 15 mints. The medium was supplemented with 3 % sucrose and gelled with 0.8% agar. Basal medium was supplemented with different concentration of growth regulators (Kin, NAA) either alone or in combination tried for shoot initiation. (Table 2). The cultures were incubated at 25 ± 2 ^{0}C with 16/18 hours photoperiod under white fluorescent tubes

Table 2 Treatments of different concentrations and combinations of hormones for multiple shoot initiation in *Chlorophytum* spp.

2.0 mg/l NAA with different concentrations of KIN	
1	2.0 mg/l NAA + 1.0 mg/l KIN
2	2.0 mg/l NAA + 1.5 mg/l KIN
3	2.0 mg/l NAA + 2.0 mg/l KIN
4	2.0 mg/l NAA + 2.5 mg/l KIN

1.4 Sub-culturing & shoot multiplication

After one week the initiated shoots were separated and sub cultured on to fresh medium. Different concentrations and combinations of hormones with MS medium were tried for multiple shoot formation (Table. 3). Minimum 6 explants were used for each treatment. Data were presented in mean number of shoot proliferated from explants.

Table 3 Different concentrations and combinations of hormones for shoot multiplication

A.2.0 mg/l NAA with different concentrations of BAP	
T_1	2.0 mg/l NAA + 0.5 mg/l BAP
T_2	2.0 mg/l NAA + 1.0 mg/l BAP
T_3	2.0 mg/l NAA + 2.0 mg/l BAP
T_4	2.0 mg/l NAA + 2.5 mg/l BAP
b. 2.0 mg/l NAA with different concentrations of KIN	
T_5	2.0 mg/l NAA + 0.5 mg/l KIN
T_6	2.0 mg/l NAA + 1.0 mg/l KIN
T_7	2.0 mg/l NAA + 1.5 mg/l KIN
T_8	2.0 mg/l NAA + 2.0 mg/l KIN
T_9	2.0 mg/l NAA + 2.5 mg/l KIN

1.5 Rooting

After two to three sub culturing, the shoots of about 5-6 cm in length were separated and transferred to the different concentrations and combinations of rooting media (Table. 4)

Table 4 Treatments for root induction

S. No.	Treatments
1	Basal MS (half) medium with 500 mg/l activated charcoal
2	Basal MS (half) medium without charcoal
3	MS (half) +0.5 mg/l IBA with 500 mg/l activated charcoal
4	MS (half) +0.5 mg/l IBA without charcoal
5	MS (half) +1.0 mg/l IBA with 500 mg/l activated charcoal
6	MS (half) +1.0 mg/l IBA without charcoal

1.6 Acclimatization of plants and transfer of *in-vitro* regenerants to field

Once the roots were formed, the plants started growing. After 15 days the *in-vitro*regenerated plants were ready to transfer to the soil. After rooting, plantlets with sufficient roots were removed from the culture medium carefully, washed thoroughly till complete media was removed from the plantlets surface. The plants were treated with 0.1 % bavistin/l and were transferred to sterile sand for 5 to 6 days. To avoid the physiological shock, the plants were transferred first to the sterilized sand for about 5 to 6 days. These plantlets were supplemented with one forth strength of major and minor MS salts solution once daily. To avoid immediate physical and physiological shocks like desiccation etc., these plants were covered with transparent polythene sheets in order to maintain minimum 60-65 % humidity. Plantlets were transferred to small pots containing soilrite and sand (1:1) and subsequently to the field.

Results & Discussion

2.0 *In-vitro* regeneration potential of *Chlorophytum* genotypes

The present investigation was carried out to standardize a protocol for high frequency *in-vitro* propagation of *Chlorophytum*. Different explants of *Chlorophytum* were exposed to number of different combinations and concentrations of phytohormones for establishing a complete protocol for the production of *Chlorophytum* plantlets *in-vitro*. The results of the present study are as follows:

2.1 Effect of surface sterilization treatments

Young buds, leaf and rhizome segments were collected from *Chlorophytum* plants and sterilized by the specific sterilants. Different concentrations and combinations of sterilant were tried for surface sterilization. Among the six treatments tested, treatment # VI gave best response for surface sterilization of explants, in which explants (2-3 cm) of *Chlorophytum* were washed with 2% (V/V) labolene for 5 min and rinsed with running tap water. Explants were dipped in 1.0% solution of bleaching powder with 4-5 drops of 0.1 percent ampicillin solution for 20 min followed by 2 % solution of bavistin for 15 min. and 0.2 % mercuric chlorite solution with 2-3 drops of tween-20 for 15 min, then at last explants were rinsed by 70% ethanol for two min. followed by several washing with sterile water (Table 5)

Table 5 Response of surface sterilization treatments for *Chlorophytum* explants

Treatment No.	Surface sterilization treatments			Response
	Sterilants	**% of sterilant**	**Time**	
I	Labolene	2.0	5 min.	80% contamination
	Sod. Hypochlorite	2.0	10 min.	
	$HgCl_2$	0.2	10 min.	
	Ethanol	70	1 min	
II	Labolene	2.0	5 min.	
	Sod. Hypochlorite	2.0	15 min.	50% contamination
	$HgCl_2$	0.2	20 min.	
	Ethanol	70	2 min	
III	Labolene	2.0	5 min.	50% contamination
	Bavistin	1.0	15 min	
	Sod. Hypochlorite	2.0	15 min.	
	$HgCl_2$	0.2	20 min.	
	Ethanol	70	2 min.	
IV	Labolene	2.0	5 min.	30% contamination
	Bavistin	1.0	20 min	
	Sod. Hypochlorite	2.0	20 min.	
	$HgCl_2$	0.2	20 min.	
	Ethanol	70	2 min.	
V	Labolene	2.0	5 min.	30% contamination
	Bleaching Powder	1.0	15 min.	
	Bavistin	1.0	20 min	
	$HgCl_2$+Tween 20 (2 drop)	0.2	15 min.	
	Ethanol	70	2 min.	
VI	Labolene	2.0	5 min.	10% contamination
	Bleaching Powder+ ampicilline (0.1%)	1.0	20 min	
	Bavistin	2.0	15 min.	
	$HgCl_2$+Tween 20 (2 drop)	0.2	15 min.	
	Ethanol	70	2 min.	

2.2 Media for shoot initiation

In a pilot experiment, all three explants, leaf, tuber part and crown of various sizes (1.0 to 2.0 cm) were subjected to the MS medium supplemented with different concentrations and combination of phytohormones. No response observed when leaf and tuber part were used as explant. Further, crown parts were used for the standardization of protocol.Among all the concentrations and combinations tested in the present study (Table 6), MS media with 2.0 mg/l NAA + 2.0 mg/l KIN was found best for

shoot initiation (Fig A) .

Table 6 Media for shoot initiation

Hormone concentration (mg/1)	No. of explant Inoculated	No. of explants contaminated	No. of explants responded	Mean no. of shoots initiated	Response (%)
NAA+KIN					
2.0+1.0	6	1	2	1.5	40
2.0+1.5	6	1	2	1.0	40
2.0+2.0	6	2	3	3.0	75
2.0+2.5	6	1	2	2.5	40

2.3 Media for shoot Multiplication

The growing shoots of 3-5 cm in length were separated from the rosettes and recultured on different media(Table 7). In this case, the separated shoots were inoculated directly as explants instead of using their young buds as explants.

The effect of different concentrations and combinations of phytohormones on shoot multiplication was examined in this experiment. The percentage response varied with the concentrations and combinations of phytohormons used in the experiment. The best growth and multiple shoots formation was observed in MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l KIN . All other treatment except, the treatments mentioned above were found to be less responsive for shoot multiplication (Fig B).

Table 7 Effect of different combinations of plant growth regulators on multiple shoot formation in *Clorophytum* genotype

Hormone Concentration (mg/1)	No. of explants Inoculated	No. of explants Contaminated	No. of explants Responded	Mean no. of shoots initiated	Response (%)
NAA+BAP					
2.0+0.5	5	0	4	3.66	80
2.0+1.0	5	0	4	3.33	80
2.0+2.0	5	0	4	4.00	80
2.0+2.5	5	0	4	3.33	80
NAA+KIN					
2.0+0.5	5	0	2	3.0	40
2.0+1.0	5	0	3	1.5	60
2.0+1.5	5	0	3	2.0	60
2.0+2.0	5	0	5	4.0	100
2.0+2.5	5	0	2	3.0	60.0

Fig. A

Fig B

2.4 Rooting

In an attempt to induce roots, micro shoots of about 5-6 cm in length were removed and sub cultured on the rooting media. About 50 plantlets were tried for root induction with different concentrations of IBA. It was observed that basal MS media with different concentrations of IBA also induced rooting but percentage of rooting and number of roots per shoot were very low (Table 8). Only fifty to sixty percent rooting was observed on basal MS medium with 2-.5 mean number of roots. Half strength MS medium with activated charcoal showed best response among all these concentrations tested. 80 % regenerated shoot-produced roots with 8.0 mean numbers of roots per shoot . The results also confirmed that activated charcoal is necessary for rooting.

Table 8 Effect of different media with IBA on root induction

Media	Rooting percentage	Mean no. of roots/ explant	Response
Basal MS (half) medium			
With 500 mg activated charcoal	80.00	8.00	Healthy roots
Without charcoal	60.00	5.00	Small roots
MS (half) +0.5 mg/l IBA			
With 500 mg activated charcoal	50.00	3.00	Normal roots
Without charcoal	60.00	1.50	Normal roots
MS (half) +1.0 mg/l IBA			
With 500 mg activated charcoal	60.00	2.00	Normal roots
Without charcoal	50.00	1.50	Normal roots

2.5 Acclimatization of plants and transfer of regenerates to field

Once the roots were formed the plants started elongation. Thus after 15 days the plants were ready to transfer in the soil. To avoid the physiological shock, the plants were not transferred to soil directly, but they were transferred first to the sterilized sand. After one week, these plants were transferred to small pots, which contain a mixture of soil + compost + sand at the ratio of 1:1:1 subsequently the plants acclimatized to semi shade condition before ultimately shifted to the field. The plant survival percentage was 80 recorded after one month of transferring to the pots .Due to large scale and indiscriminate collection of the wild material and insufficient attempts either to allow its replenishment or its cultivation *Chlorophytum* is rapidly disappearing from nature. The natural regeneration of this herb is through tuberous roots. Seed germination is only 14-16 percent.

Thus an *in-vitro* method for conservation and multiplication of this crop is very useful. High frequency *in-vitro* regeneration technology can be further exploited for obtaining somoclones with higher medicinal value. In this experiment, strong treatment has been given to explants to protect them from contamination. Tissue culture has been used to accelerate plantation development, to shorten breeding cycle and to rapid multiplication. The best use of micro propagation technique is to overcome dormancy problem. Plant tissue culture has been successfully used to micro-propagate medicinal plants.

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