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MEDICAL PROSPECTS AND MICRO PROPAGATION OF *WITHANIA SOMNIFERA* WITH TISSUE CULTURE TECHNIQUE IN LAB CONDITION

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Abstract

The vegetative micro propagation of flowering plants is mainly made by the cutting technique. However, this reproductive process not always presents satisfactory results, especially at times of the year when the plant lives an intensive vegetative development, this way affecting, its commercial production. Nowadays, the vegetative Micro propagation in ornamental plants has been represented as a propagation method that supplies a good shooting, rooting and other parts, without the necessity of a phytohormones application. The complete ringing technique was used in vegetative micro propagation of *Withania somnifera*. It was used completely randomized delineation and the data submitted to the variance analysis, by the Anova Test to a level of 5% of statistic probability. Each treatment consisted of three replications, analysed after 30 days of the installation of the experiment. It was concluded that the vegetative micro propagation practice promoted enough rooting, shooting & other plant parts in all substratums, and the organic soil the one that supplied the most development of the roots. The advantage of vegetative micro propagation are, to obtain a high degree of crop uniformity to overcome complex dormancy problem, seedling growth, low seed viability and difficulty in procedures dependent upon micro propagation. The main advantage of tissue culture technology lies in the production of high quality and uniform planting material on a year- round basis under disease-free condition anywhere irrespective of the season and weather.

Key words: Phytohormones, *Withania somnifera*.

Introduction

Withania somnifera is commonly known as Indian oleander or kaner. Which is belongs to Apocynaceae family in the genus *Nerium* also called adelfa. Oleander is a perennial, evergreen shrub or small tree and now widely distributed in most tropical and sub-tropical areas of the world as well as India Thrives in hot climates - used in landscaping and as potting plants. Leaves are simple, lanceolate, 7.5-25.5 cm long, dark green above, leathery, with a prominent midrib and veins. White, pink or red showy flowers from 2.5-7.5 cm in diameter that grow in clusters. All parts of the plant are toxic, including the dried leaves. Humans, livestock, dogs, cats, birds and horses are susceptible to poisoning from oleander. It consists of dried root of *Nerium indicum* mill (Apocynaceae), a large glabrous evergreen woody shrub with milky juice that group up to the height of 4 m and bearing leaves all the year around. *Nerium* has various medicinal uses and the leaves and flowers are thought to action as tonic, cardio tonic, Diaphoretic, Diuretic, Emetic, and expectorant. Leaves and flowers are also used to treat Malaria and as traditional medicines. it induced the termination of embryo. The tincture or decoction is been used externally to reduce swelling and Scabies. The root powder is an external remedy for Hemorrhoids and Ulcer around genitals. Leaves and bark is treated as insecticidal, rat poison and parasitic. The plant of Indian oleander is thought to possess anti-cancerous activities. In forestry, clonning of trees through tissue culture is all the more importance because of forest species are heterozygous due to their cross pollination nature, so the progenies raised through seeds are not to parent type for their qualitative and quantitative traits due to segregation. As the tree ages, it becomes very much difficult or impossible to root the cutting taken from such trees.

Present studies have demonstrated that immature seeds obtained from green pods of *Withania somnifera* a pharmaceutically valuable, ornamental, epiphytic forest orchid having horticultural importance, can be germinated asymbiotically *in vitro* for rapid micropropagation. (Murashige and skoog, 1962) containing Naphthalene acetic acid (NAA) and coconut water (CW) was found most effective for high percentage of seed germination and seedling development. The rate of survival of plantlets after the transfer from culture vessel to natural condition was 98-99%. This method can be exploited for the rapid propagation and conservation of *Withania somnifera*.

Materials and Methods

Tissue culture media

MS Media (Murashige and Skoog, 1962) was a landmark in plant tissue culture research and is the most frequently used medium for *Withania somnifera* tissue culture work. Based on its composition other media were evolved to meet the diverse experimental and species-specific requirements. There were Linsmaier and Skoog (1965), B5 Medium of Gamborg et al (1968), Nitsch and Nitsch (1969).

Medium composition

The nutrient medium for most plant tissue culture was composed of four groups of ingredients.

Inorganic nutrient

Macro elements, Micro elements

Organic nutrient

Vitamins, Amino Acids & Carbon source

Plant growth regulators

Auxins like Indole-3-acetic acid (IAA), Indole-3-Butyric acid (IBA), Naphthalene acetic acid (NAA), Dichlorophenoxyacetic acid (2,4-D), Trichlorophenoxyacetic acid (2,4,5-T),

Cytokinins, Naturally occurring cytokinins – Zeatin, Benzyl amino purine – BAP, Furfuryl amino purine – Kinetin, Gibberellins, Ethylene & Carbon and energy source (sucrose)

Gelling Agents

Agar, Agarose & Gelrite

pH of medium

The pH of medium is adjusted between 5.0 to 6.0 before sterilization. Plant cells and tissue require optimum pH for growth and development in culture.

Materials

Conical flasks, culture tubes, measuring cylinders, paradises, pipettes and culture vessels of different shapes and sizes are required in large quantities.

Autoclave

Lamina air flow bench (LAF)

pH Meter

Sterilization

Medium should be autoclaved at 121 °C for 15-20 min under 15 psi. Care must be taken while cooling the solutions. A rapid lose of pressure, exceeding the rate of reduction in temperature will make the liquid vigorously. The pressure gauge of the autoclave should be at zero before the autoclave is opened. Some of plant growth regulators urea-certain vitamins, antibiotics, plant extract and enzyme used in tissue culture is thermo labile. These compounds should not be autoclaved. There are several possible sources of contamination of the medium like culture vessels, the medium itself, the explants, the environment of the transfer area, instrument used to handle plant material during inoculation and subculture the environment of the culture room, the operator.

Sterilization of the glassware

Glass culture tubes were mostly sterilized together with the medium glassware may be sterilized by autoclaving or dry heating in oven at 160-180C for 3 hrs. but dry heat sterilization has disadvantage of poor circulation and slow penetration glassware were sterilized with medium in autoclave at 121°C for 15-20 minutes under 15 psi pressure.

Sterilization of the instruments

The instruments used for aseptic manipulations, such as forceps, scalpels, needles and spatula were normally sterilized by dipping in 95% ethanol followed by flaming and cooling. This is done at start of the transfer work and several times during the operation. The alcohol should be regularly changed as bacterial strains persist in alcohol for more than a week. Flaming of the instruments is achieved by the Bunsen burner.

Sterilization of the plant material

Surface of the plant parts carries a large part of microbial contaminant. To avoid this, surface of the tissue should be thoroughly surface sterilized before inoculating it on a medium with the sterilizing agents like calcium hypochloride, mercuric chloride, hydrogen peroxide, bromine water antibiotics etc,

Sterilization of the transfer area

It is very essential that all precautions be taken to prevent the entry of the contaminant into the culture tube when its mouth is open either for inoculating explants or during sub culturing. To achieve this, all transfer operations are carried under strictly aseptic conditions. In the tissue culture laboratory in I BAS laminar air flow cabinets are used for aseptic manipulations before performing the transfer operations are placed inside and U.V. tube light of laminar is turned on for half an hour and after that it is switched off an blower and tube light is switched on and explants is taken inside. The platform and hands of the operator should be wiped with 70% alcohol after every 2-3 transfer.

Procedure

The explants were cut and washed with tap water and liquid detergent teepol (5-10 drop/ 100 ml) then wash with distilled water to remove excess amount of detergent. Explants treated with 70% ethanol for 1-3 min. and washed it with distilled water. After this, give the treatment of fungicide (Bavastin) 5-20 min, then washed with distilled water. Now give the treatment of antibiotics (streptomycin) for 5-20 min, then washed with distilled water. Give the treatment of either HgCl₂ or NaClO for 3-5 minutes with various concentrations in the LAFB. After this washed the explants with autoclaved distilled water for 5-6 times.

Aseptic inoculation of explants for establishment of primary culture

The explants so sterilized were inoculated on to MS Media supplemented with different concentration of cytokinins and auxins in laminar Air Flow cabinet under aseptic conditions using flame-sterilized forceps. Prior to inoculation proximal and distal ends of explants were trimmed and finally 5mm-8mm long explants were inoculated on to nutrient medium. Mark each culture tube or flask with glass marking pencil. Tubes or flask were plugged with tight

fitting cotton plugs made of non- absorbent cotton. All the culture was incubated in a culture room at 23-28° C for 16 hrs. Photoperiod (2000-6000 lux)

Sub culturing of primary culture:

Sub culturing of primary culture depends on our aim.e.g. If the primary culture was callus then this can be used for various aspects of plant metabolism and differentiation such as-

(1) Nutrition of plants. (2) Cells and organ differentiation and morphogenesis. Somaclonal variation and its exploitation. (3) Developing cell suspension culture. (4) Genetic transformation. (5) For the production of secondary metabolites.

If the primary culture was shoot then it excised from explants with the sterilize scalpel and then transfer on cytokinin supplemented medium for their further development. After suitable growth, this shoot transfer on a rooting media (Mostly Auxin) then transfer in soil rite tissue culture raised plant transfer to green house for hardening and after this transplant into field.

Results and Discussion

In the present study we have evaluated the effect of BAP, Kinetin and IAA on the nodal segments of *N.indicum*. The results are presented in table 2. It was interesting to note that the 0.5 BAP + 1.5 Kinetin + 1.5 IAA hormones showed maximum advantageous effect on the callus formation followed by 1.5 BAP + 0.5 Kinetin + 0.5 IAA, where the callus formation decreased to ++ from +++. The least callus formation was observed at the concentration of 0.5 BAP + 1.5 Kinetin + 0.5 IAA.

Table: 1 Effect of BAP, Kinetin and IAA on the apical bud of *N. indicum*

S. No.	Medium	Hormone Taken (mg/L)	No of explants	No. of Shoots/explants	Callus Formation
1.	MS	0.5 BAP + 1.5 Kinetin + 0.5 IAA	15	1-2	+

2.	MS	1.5 BAP + 0.5 Kinetin + 0.5 IAA	10	1-2	++
3.	MS	0.5 BAP + 1.5 Kinetin + 1.5 IAA	10	4-6	++++
4.	MS	2 BAP + 0.5 IAA	30	1-3	++
5.	MS	2 BAP	30	1-2	+
6.	MS	1 BAP + 2 Kinetin	30	4-5	++

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table: 2 Effect of BAP, IAA on Petiole of *N. indicum*

S No	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	1 BAP + 0.5 IAA	15	2-3	+++
2.	MS	1 BAP + 1 IAA	15	4-5	++++
3	MS	1 BAP + 2 IAA	10	2-3	++

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

When hormonal combination of 1 BAP and 0.5 IAA to 2 IAA is applied on petiole of *Withania somnifera* the callus formation is decrease when amount of IAA increase.

Table: 4 Effect of kinetin on the nodal segment of *N. indicum*

S No	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	2 kinetin	30	5-6	++++

Kinetin is a cytokines, responsible for cell division and differentiation of adventitious shoot formation. When a nodal segment of *Withania somnifera* were cultured on 2mg/L kinetin supplement media, bud breaking and callus induction were observed. Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (Auxins and Cytokinins). The specific concentration of plant growth regulators needed to induce callus, varies from species to species and even depends on the source of explants.

The initiated calluses were then allowed to grow on shoot induction medium with increasing concentration of Kinetin. The nodal explants of *Withania somnifera* was produce masses of callus and shoot buds when supplemented with both auxin and cytokinines.

Cytokinin are key regulators of a large number of processes in plant development, which is highly plastic and adaptive, and remarkably resilient and self-perpetuating. Cytokinin signaling involves a multistep two-component system, also called a phosphorelay signaling system. Genes encoding the central components to build the cytokinin phosphorelay system are found in diverse plant species, resulting in the establishment of a canonical model of cytokinin signaling that is likely representative and conserved in plants. The cytokinin-signaling pathway can be modulated by nutrients, stress, and other hormones, and is integrated into a signaling network in both shoots and roots (Muller *et al.*, 2007).

Cytokinin are important adenine derivatives that serve as hormones to control many processes in plants. They were discovered as factors that promote cell division in tobacco tissue cultures and have been shown also to regulate several other developmental events. Kinetin which was isolated 50 years ago for the first time as a plant hormone, as well as other cytokinins isopentenyladenine, zeatin and benzylaminopurine induce callus (clusters of dedifferentiated plant cells) to redifferentiate into adventitious buds. Because of some similarities in the biological

phenotypes of cancer and callus cells, cytokinins and especially kinetin, affect the differentiation of human cells through a common signal transduction system. Therefore, cytokinins found their way to use in molecular medicines (Barciszewski, *et al.*, 2007 & Dobránszki *et al.*, 2010).

Our results also demonstrate that combination different doses of hormones affect the growth of different explants. This plant has different medicinal activity. If we were able to continue the invitro culture technique and grow these plants in large amount then we may be able to help the pharma industries.

Plant tissue culture is a step to increase its quantity. Plant tissue culture is a technique, which can reduce its proliferation time. This experiment was an effort to do the same. When explants were cultured on MS medium supplemented with varying concentration of cytokines, good callus response was found. The effect of plant growth regulators on callus induction and morphogenesis of plant explants were studied. Under the experimental conditions tested, BAP + Kinetin + IAA combination was the most efficient PGR combination on callus induction and shoot regeneration. The maximum callus formation from apical bud was possible in a range of 0.5 BAP + 1.5 kinetin + 1.5 IAA (Mg/L) concentrations. The highest percentage of shoot regeneration was induced on MS medium supplemented with 2mg/L kinetin.



Fig. 1 Inoculated Culture Flasks

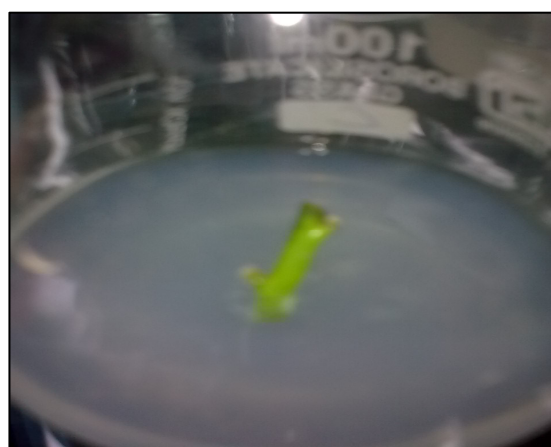


Fig. 2 Explants after 21 days inoculatio

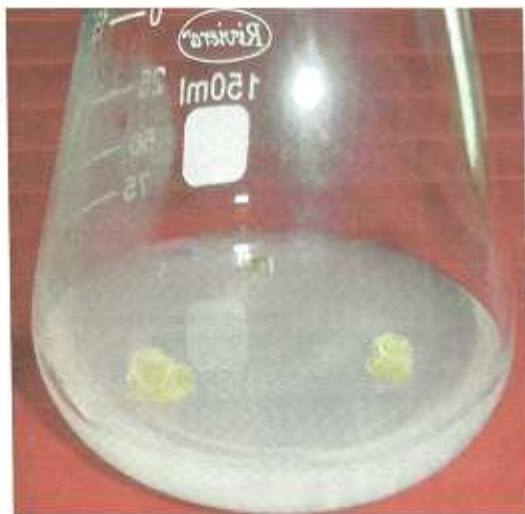


Fig. 3 Apical bud of *Withania somnifera* showing callus growth on MS medium supplemented with 0.5 BAP+1.5 Kinetin+1.5 IAA (mg/L) hormonal concentration



Fig. 4 Effect of 2 Kinetin(mg/L) on the nodal segment of *Withania somnifera*



Fig.5 Explants growth after 3 months inoculation supplemented with 2mg/L Kinetin.

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