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Micropropagation of *Azadirachta indica* and assessment its fungicidal action

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Abstract

An effective protocol has been developed for micropropagation of *Azadirachta indica* using Murashige and Skoog (MS) medium supplemented with different hormones like 1-Naphthyl acetic acid(NAA),3-Indole acetic acid(IAA),3-Indole butyric acid(IBA),6 Benzyl aminopurine (BAP),2,4- dichlorophenoxyacetic acid(2,4-D)and Kinetin. Extracts obtained were analyzed using HPTLC. Different sample preparations were compared for action against fungi like *Aspergillus niger, Penicillium sp, Aspergillus versicolor, Drechslera sp* with sample from the original plant. The present in vitro procedure is more effective than the conventional techniques used in conservation and mass propagation of this medicinal plant.

1. Introduction

Azadirachta indica or Neem as it is commonly called was referred by ancient Indian texts as "sarva roga nivarini"-The curer of all ailments. It is a large tree that can grow up to 35 meters. It has been labeled the wonder tree of the humid tropics. Many parts of neem tree have anti-microbial properties. They provide active ingredients for toothpastes medicines, cosmetics and insect repellents. Most of the antimicrobial oil from neem seeds goes into soap in India (Sai Jyothi Pattnaik, 1990). The effect of "neem oil" (prepared by aqueous extraction of neem kernels) was studied on 14 common fungi, including Trichophvtonrubrum, *T. violaceus*, *T. Concentricbus*, *T.mentagrophytes, Epidermophyton floccosum, Mierosporum citaneum, Scrophulariopsis brevicaulis, Geotrichum candidum and Fusarium sp* and found that it did not inhibit fungal growth and, in fact, the neem oil itself actually contained several species of growing fungi (Khan and Wassilew, 1986). Yet an article reported that "10% Neem oil diluted from its emulsifiable concentrate formulation" completely inhibited several species of fungi such as *Aspergillus niger, Fusarium moniliforme, Macrophomina phaseolina and Drechslera rostrata.* However, the specific details of this formulation were not provided (James C. Locke 1994). Neem shows wide genetic variability in terms of tree size, morphology, fruit size, and fruit production. They exhibit different flowering behavior and showed varied tolerance to drought, frost, and other climate stresses. Variability is due to open pollination. Seed raised crops show segregation of characters. This variability is short term. Therefore there is need to select plants with desirable characters and clonally propagate the selected trees for increasing productivity.

2. Materials and Methods (GeorgeE F, 1993),

Plant Neem plant *Azadirachta indica A.Juss* from botanical garden of WCC were used for experiments.

Microorganisms *Aspergillus niger, Penicillium sp, Aspergillus versicolor, Drechslera spp* were obtained

Equipments and chemicals

- Soxhlet apparatus, HPTLC instrument, Forceps and Scalpels
- Mercuric chloride(sterilent), 70% ethanol, Distilled water
- MS media **(**Murashige & Skooge, 1692).

Method

MS medium was prepared (after standardization) and stocked in a sterile lab. Hormones were also used in different concentrations ranging from 0.5 to 5mg/10ml (stock). pH was maintained at 5.6-5.8.Hormones used were [1-Naphthyl acetic acid(NAA), 3-Indole acetic acid (IAA), 3-Indole butyric acid(IBA), 6 Benzyl aminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin. Explants (Plant leaves, stem, meristem or root) were cut and sterilized. Sterilant was standardized. Mercuric chloride was used as sterilant (in different concentrations).

- Explants were cut into small pieces using sterile scalpel and was put in 70% alcohol
- The cut explants were then surface sterilized with (0.1 %)$HgCl_2$ and washed with sterile water dried using filter paper before explants were inoculated into medium in test –tubes and incubated in adequate light and temperature.Explants were subjected to a photoperiod of 12 hours.

Extraction

Callus was weighed and subjected to Soxhlet extraction.

- Solvent used-diethyl ether
- Extraction time-2hrs,4hrs,6hrs

Analysis

Part of the sample was analyzed using HPTLC .Pre coated silica plates were used and

Solvent used was ethyl acetate: hexane (20:80).Two spots were found in the ether extracted sample and ethanol extracted sample (the callus samples extracted by soxhlet and by ethanol) Samples were compared with known standards (at 360nm, 254nm).

Fungicidal action

Different fungi like *Aspergillus niger, Penicillium sp, Aspergillus versicolor, Drechslera sp* were cultivated on potato dextrose agar. Mueller Hinton agar was prepared and fungi were spread with sterile cotton swabs into petriplates with the medium. Two wells were bored. The plant extract was added to one well. The callus extract was added to the other well. Callus sample extracted with different solutions like water, alcohol and methanol were used and the fungicidal action was compared after incubation.

3. Results

Various hormones used showed different types of response on different explants (Table I). The standardization of the concentration of the sterilant was done and the best concentration was 60 sec in 0.1% merrcuric chloride (Table II). Effect of various hormones like NAA, Kinetin and IBA cause callus in 2-3 weeks while combination of hormones caused shooting but rooting was found with only IAA (Table III- X). The extract did show fungicidal action against *Aspergillus niger, Penicilliumspp, Aspergillus versicolor* and *Drechslera* spp. Inhibition zone of callus extracted by ethanol against *Aspergillus niger* was found to be maximum (Table XI-XV).

Table I Effect of Hormones

Hormones	Result (explant used)
NAA	Callus (stem)
IAA	Callus and Root (leaves)
IBA	Callus (leaves)
NAA + KINETIN	Callus after 3 weeks (leaves)
KINETIN	Callus after 2 weeks(leaves)
NAA+IAA	Shoot(from stem)
BAP+IBA	Shoot(from stem)
NAA+IBA	Callus, Shoot after 2 weeks (stem and meristem)
BAP	Shoot (leaves)
2,4-D	Very little callus(leaves)
IAA + BAP	Callus(leaves and stem)
NAA + BAP	Shoot and callus(stem and meristem)

Table II Effect of mercuric chloride (0.1%)

Time (sec)	Control of contamination
30	50%control
60	90%control

Table III Effect of NAA

NAA(mg/l)	Number of days	Callus formation
1	10	nil
2	8	+
2.5	7	+
3.0	4	+
4.0	4	+

Table IV Effect of IAA

IAA (mg/l)	Number of days	Callus/root formation
1.0	14	nil
2.0	14	+
3.0	7	+
4.0	4	+

Table V Effect of IBA

IBA (mg/l)	Number of days	Callus formation
1.5	14	nil
2.0	15	+
3.0	7	+
3.5	3	+

Table VI Effect of BAP

BAP(mg/l)	Number of days	Shoot formation
0.1	20	nil
2.0	20	+
3.0	7	+
4.0	4	+
5.0	4	+

Table VII Effect of BAP & IBA

BAP + IBA (mg/l)	Number of days	Shoot formation
0.1 + 0.1	15	nil
1.0+ 1.0	14	+
2.0 + 2.0	7	+
2.5 + 2.5	4	+

Table VIII Effect of NAA and Kinetin

NAA + Kinetin (mg/l)	Number of days	Callus formation
0.5 + 0.5	15	nil
1.0 + 1.0	15	nil
1.5 + 1.5	17	nil
2.0 + 2.0	20	+
2.5 + 2.5	20	+

Table IX Effect of NAA & IAA

NAA + IAA(mg/l)	Number of days	Shoot formation
0.1+ 0.1	14	nil
0.5 + 0.5	15	nil
1.0 +1.0	15	+
1.5 + 1.5	7	+
2.0 + 2.0	4	+
2.5 + 2.5	4	+

Table X Effect of NAA &IBA

NAA + IBA (mg/l)	Number of days	Shoot formation
0.1 + 0.1	20	nil
1.0 + 1.0	14	nil
1.5 + 1.5	14	+
2.0 + 2.0	14	+

Comparison of fungicidal action of callus extract solutions with leaf extracts:

Table XI Fungus used: Aspergillus versicolor

Contents in well	Zone of inhibition (mm)
Callus extract	13
Leaf extract	10

Table XII Fungus used*: Aspergillus niger*

Contents in well	Zone of inhibition (mm)
Methanol extracted callus	12
Leaf extracted with methanol	5
Ethanol extracted callus	20
Leaf extracted with ethanol	5
Sterile water extracted callus	10
Leaf extracted with sterile water	0

Table XIII Fungus used: *Drechslera sp*

Contents in well	Zone of inhibition (mm)
Callus extract	7
Leaf extract	.3

Table XIV Fungus used*: Penicillium* spp

Contents in well	Zone of inhibition (mm)
Callus extract	6
Leaf extract	3

Photo 1-Effect of BAP&IBA on stem cuttings of *Azadirchta indica*

Photo 2-Effect of soxhlet extracted callus of *Azadirchta indica* on *Aspergillus niger* cultures.Callus extract on top, leaf extract at the bottom near to finger.

4. Discussion

MS medium supplemented with a combination of NAA and IBA gave rise to callus within the first week and shoot within two weeks. Combination of IBA and BAP (3-5mg/l) gave rise to shoot after 4 days. Callus was obtained when NAA was used with leaf. BAP & NAA when supplemented in medium in which stem and meristem cuttings were inoculated gave rise to callus and shoot. Leaf and stem explants gave good results and similar results were found in neem earlier by others also (Reddy et al, 2006). Alternating dark periods were found to be effective. Callus growth was found to be delayed when photoperiod of more than 12 hours was observed. Temperature was maintained at 23± 1ºC, temperature below 21ºC was found to be delaying the growth of callus. Callus, root and shoot formation was best obtained under these conditions of 23± 1ºC and 12 ours of light and such reports have been there earlier (Eeswara, 1998). The weight of callus was found to be reduced after extraction by 0.0002g.Extracts obtained from soxhlet extraction of callus were analysed and it was observed to be matching with compounds in leaf extract .They had similar Rf values (Kausik Biswas, 2002). The anti fungal activity of neem is very prominent against *Aspergillus niger* in comparision to others and stands by reports which say that antifungal properties found in neem (Varma , 1976).

5. Conclusion

Even though Neem is an indigenous and multipurpose tree having immune potentialities, it has been neglected by plant scientists in India no serious attempts have been made to conserve its germplasm and study this tree systematically until the beginning of 20th century. A successful method to extract pure compounds which can be used for specific purposes like pesticides, medicines, cosmetics, etc was attempted.

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