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Phytochemical and Antimicrobial Evaluation of *in vivo* and *in vitro* Regenerants of *Trachyspermum ammi* Linn.-A Herbal Spicy Medicinal plant

Anand Prakash, Rajeev Kumar, Bhawna, Nishi Kumari, Anupma Kumari and Santosh Kumar*

UGC Centre of Special assistance, DRS Department of Botany,
B R A Bihar University , Muzaffarpur 842001

Abstract
Investigation on the photochemical and antibacterial properties of ethanol extracts of in vivo grown plants as well as in vitro generated plantlets of Trachyspermum ammi was carried out. Nodal explants of TA when placed on MS medium supplemented with 6-Benzyl amino purine (BAP) at 4µg/ml resulted into multiple shoots. The success rate was found to be 95%. These shoots, on transfer developed bunch of branched roots in presence of Indole-3-butyric acid (IBA) at 2µg/ml and BAP at 1 µg/ml. In order to study the phytochemical properties of both in vivo and in vitro plants Trachyspermum ammi, ethanolic extracts were prepared by soxhlet extraction method for this study. The results revealed the presence of flavonoids, terpenoids, saponins, phenols and tannins. Further the ethanol extracts of such regenerated in vitro plants along with that of natural in vivo garden plants on comparison were found to be more effective against gram positive bacteria when compared to gram negative bacteria.

Keywords*: Trachyspermum ammi, Ethanol extract, Phytochemicals, Antimicrobial, in-vitro explants.*

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I. Introduction

Medicinal plants are potential sources of drugs and are used to treat serious diseases especially in developing countries[1]. The plant, chosen in the present study *Trachyspermum ammi* (Fam. Apiaceae or Umbelliferae) is one of the well known herbal and spicy medicinal plant commonly known as ajowan, bishop's weed, ajwain, ajowan caraway, carom seeds or thymol seeds[2]. This plant is believed to have originated in the Middle East, possibly in Egypt. In India, ajwain producing states are Rajasthan and Gujarat. *T ammi* is a small annual shrub which is practiced to cure the human ailments such as bronchitis, cold cough, influenza, asthma and gastro intestinal disorders due to the main active component, thymol. In Unani system of medicine, ajwain is used as a crude drug to enhance body resistance and is prescribed in amoebiasis. There is a need for selectively acting antimicrobial agents capable of inhibiting the growth of potentially pathogenic microorganisms, while not negatively impacting the bulk gastrointestinal tract microflora[3].

T. ammi is pungent and bitter, spasmodic, germicidal, antiseptic digestive, antipyretic, expectrorant and an extra ordinary tonic. The herbal plant is a source of the valuable antiseptic thymol, a stearoptene which is contained in the oil yielding seeds to the extent of 20 or 30%. Thymol has application in toothpastes and perfumes[4]. The plant is conventionally propagated through seeds. The seeds are used to flavour bread and local alcoholic drinks and also given to lactating mother as supplementary food. The technique of tissue and organ culture is being used for rapid multiplication of elite plants. In comparison to conventional propagation, micropropagation has the advantage of mass scale propagation in limited time and space, maintenance of disease free germplasm and round the year propagation of quality planting material[5].

Medicinal plants are of great importance to the health of individual and communities. The health benefits are mainly because of various phytochemicals that produce a definite physiological action on the human body. The most important of these chemically active (bioactive) constituents of plants are: alkaloids, tannins, flavonoid and phenolic compounds[6]. Many of these indigenous medicinal plants are also used for medicinal purposes[7]. Sufficient studies are required on the phytochemical and antibacterial evaluation of *Trachyspermum ammi*, hence the present work was envisaged.

II. Material And Methods

The plant, *T. ammi* grown (Fig.1A) in the SAP garden of DRS Department of Botany, B R A Bihar University, Muzaffarpur, established under UGC-SAP Scheme was used as experimental material. Nodal segments with shoot tips (2-3 cm) were excised from young plants and used as explants in the experiments. The explants were washed with 5% (v/v) teepol solution for 10 min, surface sterilized with 0.2% $HgCl_2$ for 2-3 min and rinsed 3-4 times with sterile double distilled water. Explants cultured with solid MS medium[8] containing 0.8% agar, 3% sucrose and supplemented with different concentrations of auxin and cytokinin. The pH of each medium was adjusted to 5.8 before the addition of agar and autoclaving at 121^0C. The cultures were maintained at 25 ± 2^0C. Subculture of *in vitro* shoots was carried out at periodical intervals of 4 weeks using MS medium supplemented with different concentrations of BAP either alone for shoot proliferation and in combination with IBA for root generation. The number of shoots produced after subculture divided by number of shoots inoculated was regarded as rate of multiplication[9]. The shoot multiplication experiments have been regularly conducted and maintained for over one year now.

In vitro* cultures of *Trachyspermum ammi:

Multiple shoots were regenerated on MS medium supplemented with different concentrations of BAP (1-5 µg/ml). At every interval of 4 weeks, observations in terms of number and height of shoots were recorded. All the cultures were grown under a photoperiod of 16 hrs (illuminated 1200 lux)[10]. Rooted *in vitro* shoots were taken out from the tubes and washed with distilled water to remove the agar medium and transferred to soil with vermiculite in 1:1 ratio in sterilised pots and these pots were shifted to highly humidified acclimatized room for hardening for another 20 days and then planted in the field[11]**.**

Preparation of ethanol extract of *Trachyspermum ammi:*

Plantlets of ***T ammi***, generated through both *in vivo* and *in vitro* systems were shade-dried and pulverized. The powder was treated with petroleum ether for de-waxing and removal of chlorophyll. Later, it was packed (2 g) in a Soxhlet apparatus and subjected to hot continuous percolation for 12 h by using 250 ml of ethanol (95% v/v) as solvent[12]. The extract was concentrated to dryness under reduced pressure in rotary evaporator and dried in a desiccator. These residues were used for preliminary phytochemical screening and antibiotic activities of secondary metabolites.

Phytochemical Screening

Test for Alkaloids

Five ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

Test for Amino acids

One ml of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour shows the presence of amino acids.

Test for Anthraquinones

Five ml of the extract solution was hydrolysed with diluted Conc. H2SO4 extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

Test for Flavonoids

To one ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.

Test for Glycosides

The extract was hydrolysed with HCl for few hours on a water bath. To the hydrolysate, 1ml of pyridine was added and a few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

Test for Saponins

The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam showed the presence of saponins.

Test for Steroids

One ml of the extracts was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

Test for Tannins

To five ml of the extract a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicates the presence of tannins.

Test for Triterpenoids

Ten mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml ofConc.H2SO4. Formation of reddish violet colour indicates the presence of triterpenoids.

Determination of Antibacterial activity:

The antibacterial activity of extracts against the bacterial strains viz., *Escherichia coli* MTCC 64, *Enterobacter aerogenes* MTCC 111, *Staphylococcus typhi MTCC67, Klebsiella pneumoniae* MTCC 39, *Pseudomonas aeruginosa* MTCC 424, *Bacillus subtilis* MTCC 121, was tested by agar well diffusion method[13] and zones of inhibition were measured. The extracts were diluted in dimethylsulphoxide (DMSO). Pure DMSO was taken as the control. The experiment was performed in triplicate. Microbial growth was determined by measuring the diameter of the zone of inhibition and the mean values were calculated.

III. Results

Nodal segments of the freshly collected plant, *T ammi* (Fig. 1B), were examined for their response to different combinations of phytohormones. MS medium supplemented with various growth regulators like 6-Benzyl amino purine [BAP], Naphthelene acetic acid [NAA] and 2,4-Dichlorophenoxy acetic acid [2,4-D] either singly and/or in combination, at different concentrations were used for shoot regeneration from nodal segments and Indole-3-butyric acid [IBA] for root regeneration. However, BAP (2-5 mg/l) was found suitable for rapid multiplication of shoots (Fig., 1C-E). Shoot formation was not found on 1 mg/l BAP. The highest percentage (95%) of shoot formation was obtained on 4 mg/l BAP alone and maximum number (7.8±0.5) of shoots was observed (Fig., 1F; Table 1). 5 mg/l BAP reduced the percentage (92%) shoot formation and lowered the number of shoots (3.2±0.3). Results revealed that the rate of increasing the height of shoots was very slow and these were (0.97±0.03), (1.00±0.1), (2.00±0.4) and (1.00±0.00) on 2, 3, 4 and 5 mg/l BAP, respectively. MS medium supplemented with 2mg/l IBA and 1mg/l BAP was found to be effective in percentage (100.00±0.00) root formation whose nature was of bunch and branched (Table 2).

At the same concentration, shoots were obtained deep green colour and retain the colour till the hardening of plants in the field (Fig. 1G). At highest concentration of IBA(3.0mg/l) and BAP(2.00mg/l) the percentage (92.00±0.50) formation of root was almost good but the nature of roots was changed, it was branched not in bunch(Fig. 1H) .This concentration bifurcated roots from each other and facilitated the separation of complete plantlets for hardening (Fig. 1I) 1 mg/l IBA and 0.5 mg/l BAP was proved to be less effective for percentage (42.74±1.00) root formation and the nature of root was observed to be feeble. Among size plantlets, only one became died and rest five were well survived in nature (Fig. 1J).

Table 1- Effect of BAP alone on shoot formation form nodal segment of *T. ammi* after 25 days of MS medium containing 0.8% agar and 3% sucrose.

Growth regulators (mg/l) BAP	Shoot formation (%)	No. of shoots (per explant)/	Height of shoots (cm)
1	-	-	-
2	80	3.5±0.5	0.97±0.03
3	82	4.5±0.2	1.00±0.1
4	95	7.8±0.5	2.00±0.4
5	92	3.2±0.3	1.00±0.00

Table 2 - Effect of IBA in combination with BAP on root formation and shoots survivality

Growth regulators (mgl^{-1})		Root formation [%]	Nature of roots	Nature of shoots
IBA	BAP			
1.0	0.5	42.74±1.00	Feeble	Light green
2.0	1.0	100.00±0.00	bunch and branched	Deep green
3.0	2.0	92.00±0.50	Branched	Deep green

Table 3: Phytochemical and antibacterial tests for the presence of active constituents in *in vivo* garden plants and *in vitro* generated plants of *T ammi:*

S N	Compounds	Ethanol solvent	
		In vivo grown plants	*In vitro* generated plants
Phytochemical Constituents			
1	Alkaloids	+++	++
2	Amino acids	+++	++
3	Anthraquinones	++	+
4	Flavonoids	+++	++
5	Glycosides	+++	++
6	Saponins	+	+
7	Steroids	+	-
8	Tannins	++	-
9	Terpenoids	+	+
Antibacterial activities		Zone of Inhibition (mm)	
1	*B subtilis*	14	12
2	*P aeruginosa*	00	00
3	*S typhi*	18	16
4	*E aerogens*	22	18
5	*E coli*	16	12
6	*K pneumoniae*	18	12

+++ = High; ++= Moderate; + = Low; - = absence

The results of phytochemical screening of ethanol extracts of *in vivo* grown and *in vitro* generated plants of *T ammi* are presented (Table 3). Alkaloids, amino acids, anthraquinones, flavonoids and glycosides were found in both samples and the amounts were also sufficient. Tannins and Saponins were found only in *in vivo* plants but not in *in vitro* samples whereas terpenoids were detected in feeble amount in both the samples.
The ethanol extract from both *in vivo* and *in vitro* generated plants of *T ammi* when tested against microbes, only *Pseudomonas aeruginosa* was found to be resistant to the tune of more than 50 µg/ml whereas other bacterial strains, viz., *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Bacillus subtilis* responded to the ethanol extracts of both *in vivo* grown as well as *in vitro* generated plants (Table 3). Zone of inhibition showing antimicrobial activity was determined by the agar well diffusion method.

IV. Discussion

Plant tissue culture techniques were employed to develop *in vitro* multiple shoot regeneration through direct organogenesis. Since the requirement of the experiment was to obtain ethanol extract of *in vitro Trachyspermum ammi* plant, no attempt was taken to develop callus and/or callus mediated plantlets. Further studies are required to detect the pharmacological activities of the plant and studies required to characterize the bioactive compounds in both *in vivo* and *in vitro* sources.

The results clearly gave an edge to the *in vivo* plants as compared to the *in vitro* plants, so far the antimicrobial activity was concerned[14]. But both types of extract were found to be more effective against gram positive then gram negative bacteria[15]. The decreased level of antibacterial activity in *in vitro* plantlets suggests that some of the chemicals are either lost or may have transformed in other active compounds[16]. Herbal medicines as that of *T ammi* are assumed to be harmless, nevertheless, herbal extracts need to be assured for its quality control and efficacy for a particular dose[17].

Extracts used in our study exhibited variation in effectiveness towards antibacterial property against the tested bacteria[18]. This antibacterial activity may be due to the active compounds that are present in the plant extracts[19,20]. However, some plant extracts were unable to exhibit antibacterial activity against tested bacterial strains. These bacterial strains may have some kind of resistance mechanisms, for example, enzymatic inactivation, target site modification and decreased intracellular drug accumulation[21].

V. Conclusion

Such herbal extracts since contain different phytochemicals with biological activity that can be of valuable therapeutic index. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. A preliminary screening and more research has to be undertaken to explore the wonderful therapeutic properties of these phytoconstituents having antibacterial activities..

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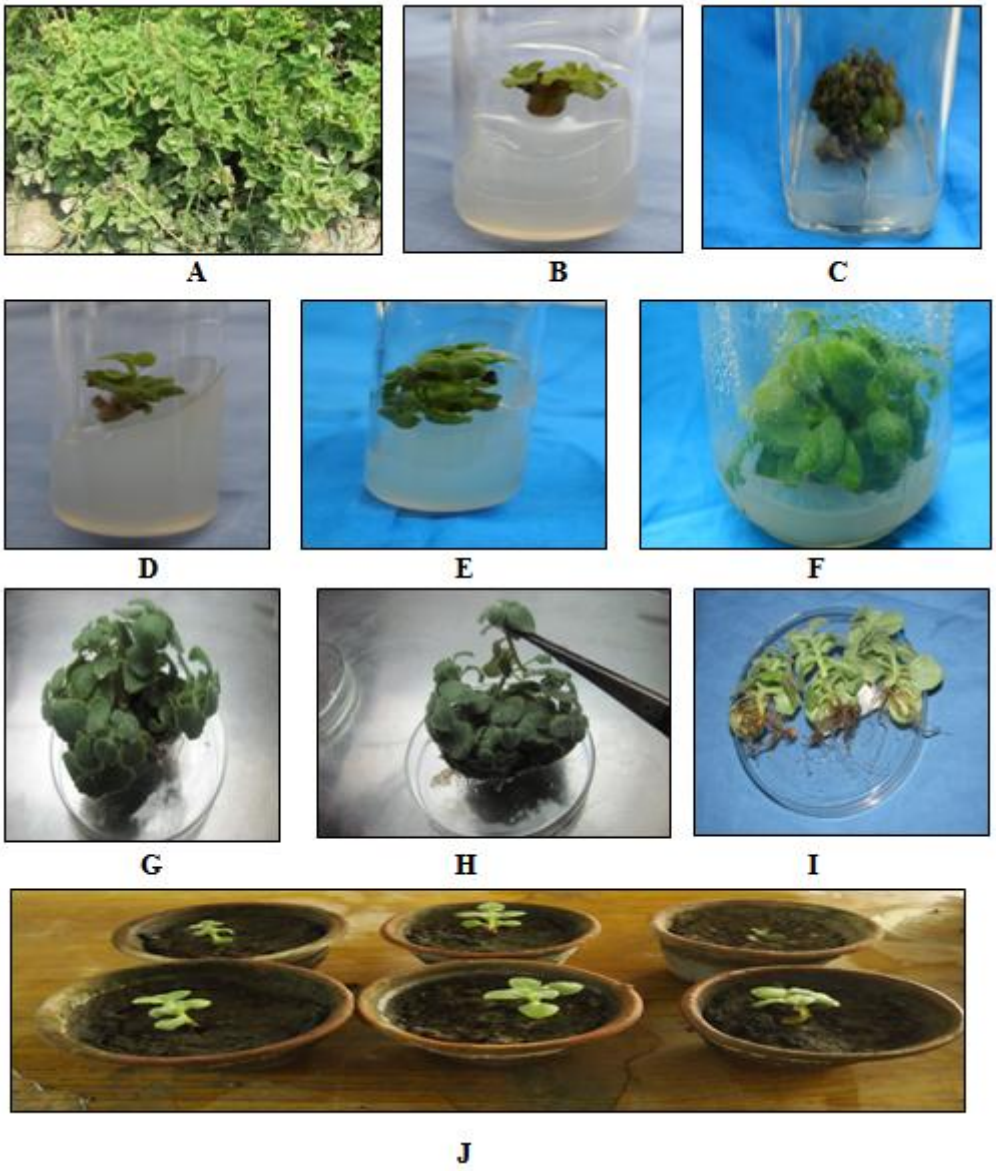

Fig. 1: A = *Trachyspermum ammi:* Herbal plant grown in SAP garden of Department; B = Nodal explants on MS medium; C –F = Development of shoot from nodal explants on MS medium with 4 mg/l of BAP: mark green & healthy shoot after 30 days of culture; G-H =: Emergence of roots after transfer on MS medium with 2 mg/l IBA and 1.0 mg/l BAP; I = Separation of twigs for phytochemical analysis and J = Rest of the twigs transferred on sterilized pots having soil & vermiculite and kept in humidified acclimatized room for hardening for another 20 days

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