ROLE OF BIOTECHNOLOGY IN MEDICINAL AND AROMATIC PLANTS

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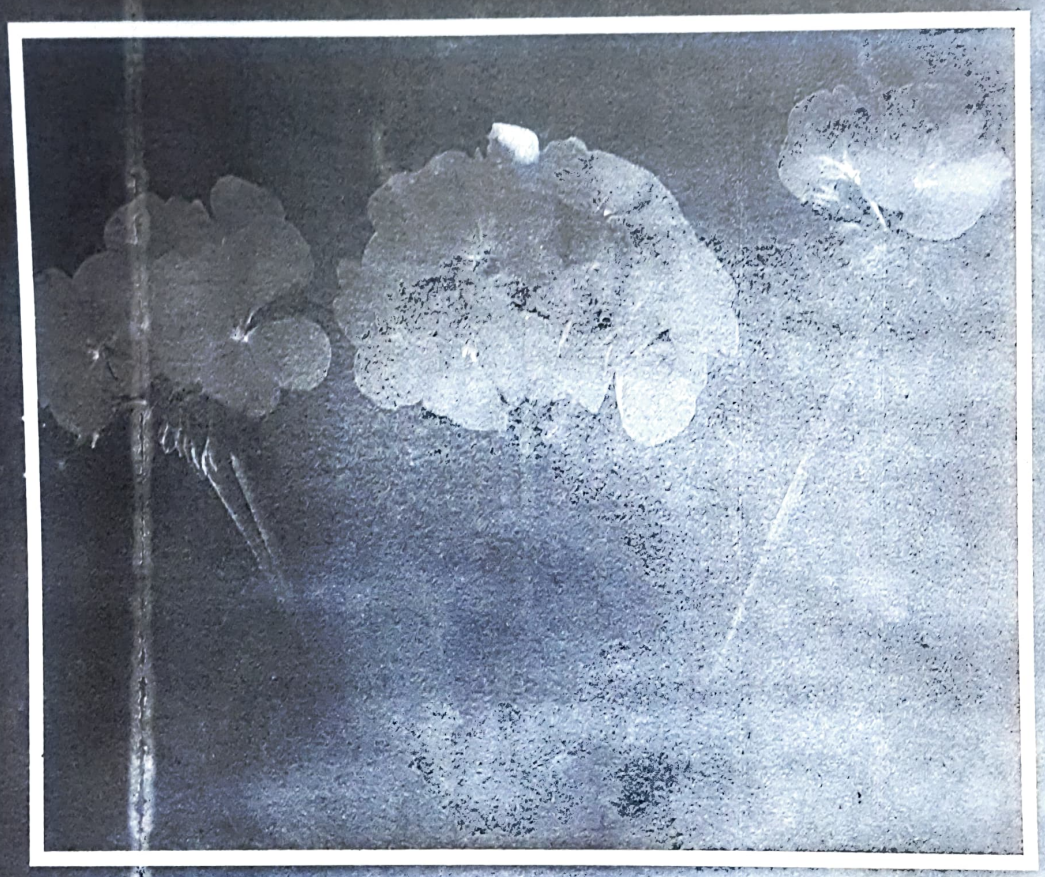

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Volume -IV

Editors

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VOLUME-IV : CONTENTS

IN VITRO PERSPECTIVES OF THE HEALING HERBS *CUMINUM CYMINUM* AND *ACHYSPERMUM AMMI*

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Chapter outline

1. INTRODUCTION :

The foundation of life was laid with the emergence of plants as the living in habitants during the volcanic age. Plants further enabled the advent of present life forms by altering the O_2 level in the atmosphere. Sustainment of these forms was ensured by the basic food products supplied by the plants. Thus, plants form the life line of the spectrum of fauna residing our mother earth. This harmonious interaction between the flora and fauna could be well enunciated by a tamil proverb which goes as follows:

"No fodder, no cattle; no cattle, no manure; no manure, no crop"

Besides providing the basic amenities of life to man, nature also keeps in her folds luxuriant herbal cover in deep ravines, nooks, corners, rock ledges *etc.* which possess medicinal properties. This also confirms on older saying 'For every disease that arises on this planet, plants or herbs give a cure" (Karnick and Pathak, 1983).

It is thus clear that primitive human societies largely depended on natural forest wealth for food, medicine, shelter *etc* (Gunther, 1945; Tarafdar, 1986; Laxman and Narayanan, 1988; Nazaruddin *et al.*, 1996). In fact, all the initial knowledge about the beneficial and curative properties of plants was the outcome of early human's quest to find herbs useful for satisfying his thirst, hunger and for the treatment of his injuries or illness (Pandey, 1989; Jain, 1994).

The tribal people and aborigines all over the world used and still use an enormous range of wild plants for their basic needs, sustenance and livelihood. There exists a direct relationship between these people and plants; study of such a relationship has been termed as 'Ethnobotany' (Maheshwari, 1983).

It is thus obvious that ethnobotany is the study dealing with all the aspects and relationships between man and plant resources. This makes it an interdisciplinary science drawn from different aspects of anthropology, botany, archaeology, ecology, economics and medicine.

Right from the ancient times people in India have been using herbal medicines, this is well accounted even in historic scriptures. Even today, years after the introduction of modern medicine in our country, the Indian system of folk medicine continues to provide medicinal relief to nearly 80% of our rural people. Remedy through herbs is a unique but genuine and

scientific healing system. Simplicity is one of the most reasonable features of this system of treatment with very little danger of any side effects where patient is cured in a smooth and natural way (Saxena and Tripathi, 1989).

The medicinal properties of these plant parts are due to the presence of certain chemicals with some definite physiological action on the human body system. Hence, in recent years the significance and awareness about medicinal plants has increased beyond mere anthropological curiosity (Bickmann, 1984).

It has been a well known fact that all systems of traditional Indian medicine have their roots in one way or the other in folk and household remedies. In addition, many modern drugs are derived from natural plant products or are chemical simulations of such substances. Therefore, the present times are witnessing revival of traditional drugs and knowledge of the medicinal plants.

However, the dictum – "Necessity is the mother of invention" fully applies to the circumstances of rural or primitive societies, which have to discover solutions to almost all their needs and problems from the natural resources around them. The plants used by them for a variety of purposes may not always be the most suited but they are the best available locally. The study of indigenous home remedies can thus serve to validate and enhance existing local uses and to provide clues to remedies having world wide potential.

Two such locally available herbs constituting an integral part of our diet, possessing healing potentiality to cure different ailments are

- ***Cuminum cyminum***
- ***Trachyspermum ammi***

Their distribution, taxonomic description and medicinal properties have been mentioned below:

2. CUMINUM CYMINUM :

2.1. Distribution : *Cuminum cyminum*, commonly known as cumin or Jeera belongs to family Apiaceae. It is a native of upper Egypt, Turkestan and the Eastern Mediterranean, where it has been used since the earliest times. It is grown chiefly in Iran, India, Morocco, China, Southern Russia, Indonesia, Japan and Turkey. Iran is major exporter of 'green cumin' (Pruthi, 1976), where it is mainly cultivated in the Khurasan province. In India, it is cultivated in almost all states (except Assam, Kerala and West Bengal) and it also stands among chief exporters. During past four-five years Iranian and Indian cumin is continuously being popularised among other countries. The chief producers of cumin in India are Uttar Pradesh, Rajasthan, Gujarat and Tamil Nadu. Growing regions for cumin in Rajasthan are Jaipur, Jodhpur, Pali, Jalore, Barmer, *etc.*

The dried fruit of the plant (*i.e.* cumin seed) is a well known condiment and spice. The physico chemical composition of the seed is, moisture 6.2%, carbohydrates 35.5%, protein 17.7%, fat 23.8%, crude fibre 9.1%, mineral matter 7.7%, Ca 0.9%, P 0.45%, Fe 0.48%, Na 0.16%, K 2.1% and vitamin B (vit B_1, B_2, Niacin), C and A. The seed contains fixed oil which is greenish brown with strong aromatic flavour (10%) as well as volatile oil (2 to 4%). The volatile oil gives a strong distinctive pleasant odour with a warm and somewhat bitter taste. The chief content of volatile oil is cuminaldehyde (20-40%). Cumin seed is an important ingredient used for flavouring soups, pickles, seasoning breads and cakes. In India, the seeds have long been employed as a stimulant, carminative and stomachic. Besides, it provides astringent and cooling effect. It is used in veterinary medicine also. Cumin is also reported to posses antifertility activity (Kant *et al.*, 1989). The essential oil has been reported to possess

antimicrobial activity (Menghini *et al.*, 1987). The oil is also employed for manufacturing liquors and cordials. Residue left after volatile oil extraction is used in soaps and fat industries.

2.2. Taxonomy : Cumin is a small, low growing, annual, aromatic herb, height of the plant reaches to 0.3m, the plants have sparingly branched tap root system, stem in much branched and angular, leaves are exstipulate, alternate with lamina deep green and highly dissected (decompound), bearing elasping (sheathing) base. Inflorescence appears about 60-66 days after germination. Flowers are borne terminally on stems. They are white coloured and arranged in compound umbels. Each umbellet has involucre of small bracts and consists of six florets of 3-5 form fruits. Each floret is pedicellate, zygomorphic due to unequal length of sepals, often bisexual and epigynous. Androecium comprises of 5 polyandrous antisepalous stamens bearing long filaments and dithecious anthers. Gynoecium bicarpellary, syncorpous, ovary inferior, bilocular, one pendulous ovule in each locule in axile placenta and is terminated by two styles and stigmas on the stylopodium. Fruit is schizocarpic cremocarp, having hairy ridges, particularly the secondary ridges; 6 vittae or oil tubes, strong heavy odour. Each mericarp contains a single seed.

2.3. Medicinal uses :Healing power and curative properties : The fruit is a rich source of thymol. Thymol is used as an anthelmintic against hookwarm infections and also as an antiseptic in many proporietary preparations. It is a stimulant, which increases the secretion and discharge of urine and relieves flatulence. It strengthens the functions of stomach and arrests any bleeding.

Digestive disorders : Cumin seeds are very useful in digestive disorders like biliousness, morning sickness, indigestion, atonic dyspepsia, diarrhea, malabrorption syndrome, and flatulent colic. One teaspoon of cumin sees is boiled in a glass of water and the decoction mixed with one teaspoon of fresh coriander leaf juice and a pinch of salt. This decoction can be taken twice daily after meal as a medicine for diarrhea.

Constipation : Cumin fruits are very useful in constipation One teaspoon of cumin fruit and one teaspoon of khirni are crushed, ground and mixed with half cup water. The patient is allowed to drink it. This decoction can be taken once a day only. Half teaspoon of mustard seeds and half teaspoon of cumin seeds are ground together and the mixture is taken with water. It can be taken once a day, till the symptoms disappear.

Cholera : Cumin powder mixed with mishri and curd is useful in cholera. Half a teaspoonful cumin powder and one teaspoon mishri are mixed in half cup curd. Thereafter, it is given to the patient to eat once daily.

Insomnia : Cumin is valuable in relieving sleeplessness. A teaspoon of the fried powder of cumin seeds mixed with the pulp of a ripe banana can be taken at night to induce sleep.

Renal colic : Black cumin seeds mixed with caraway seeds and black salt is useful in renal colic. About 20 gms of cumin seeds, 12 gms of caraway seeds and 6 gms of black salt are ground together and mixed with a little vinegar. This mixture can be taken in doses of 3 gms every hour till relief is obtained.

Common cold : Dilute cumin water is an antiseptic beverage and very useful in common cold and fevers. To prepare cumin water, a teaspoon of cumin is added to boiling water, which is allowed to simmer for a few seconds and set aside to cool. If the cold is associated with sore throat, a few small pieces of dry ginger should be added to the water. It soothnes throat irritation.

Secretion of breast milk : A decoction of cumin seeds mixed with milk and honey, taken once daily during the entire period of pregnancy, helps the healthy development of the foetus, eases child-birth and increases the secretion of breast milk.

Rheumatism : The oil extracted from the seeds is beneficial in the treatment of rheumatic and neuralgic pain.

Earache : About half a teaspoon of the seeds is heated in 30 ml of milk till the essence of the seeds permeate the milk. The milk is then filtered and used as ear drops.

Scorpion bite: One teaspoon Ajowain is ground mixed with water and put over the affected area.

Fever with abdominal disorder : 2 gms Ajowain, one cup seeds of bajra and 2 gms leaves of chircheta are boiled in water and strained. This is given to the patient to drink. It is to be given three times a day for three days.

Gastric problem : One teaspoon ajowain, one teaspoon harad and 200 gms flesh of leaves of gwarpatha are mixed together and dried in shade. It is then crushed, ground and made into powder one teaspoon of the powder is given every day till the symptoms disappear.

Loss of appetite : Ajowain is used as appetiser. One teaspoon ajowain is to be immersed in the cold water overnight in an earthen pot. It is to be taken morning and evening, twice daily for one month.

Menstruation problems : One teaspoon ajowain is crushed, ground mixed with one table-spoonful *ghee* and *bura* which is given to the patient to eat. It is to be given for three days.

Chest pain : One teaspoon of ajowain is taken in a cup of water and boiled till the volume reduces to half cup. Two tablespoon jaggery is added to it and mixed well.

Other uses : Aphrodisiac: Ajowain seeds combined with the kernel of tamarind seeds are an effective aphrodisiac. These should be fried in equal quantity of pure ghee, powdered and one teaspoon of this powder, mixed with a tablespoon of honey, taken daily with milk before retiring, makes an excellent aphrodisiac.

The greyish brown fruits or seeds are used as a spice, in flavouring numerous foods, as antioxidants, preservatives and in medicine.

Although, the above mentioned crops are easily available and grow in abundance, however, owing to their economic and medicinal value they emerge as potent candidate crops to be investigated *in vitro*.

Over the years considerable progress has been made in scientific field and tissue culture methodology is increasingly being applied for propagation of crops as a supplement to the conventional method. Whereas classical methods take long to introduce the desirable traits in the crop, the technique of plant tissue culture is decidedly superior which is now being engaged in a number of industries like agriculture, horticulture, silviculture, pharmaceutics *etc.* Besides production of a large number of plants in considerably lesser time, yet another desirable practical utility of plant propagation *in vitro* is in obtaining disease free plants. Besides this cell and tissue culture techniques offer ample scope of generating desired heritable variations in the regenerants (Batra, 1995). Above all cell cultures provide simple system which is more convenient in handling as compared to field grown plants.

These beneficial factors substantiate the use of modern techniques like tissue culture for obtaining quantitatively and qualitatively superior plants. Therefore, the tools of plant cell culture are increasingly being applied to a wide range of biotechnology ventures and in particular to the clonal propagation and genetic improvement of crop plants. For this the approaches and methodologies must be specifically adopted to the differing problems and potentialities of each crop and to the varying responses of plants.

The following text presents a summarised account of the experimentation conducted in the two candidate crops in the authors' lab. These experiments ultimately led to the establishment

of efficient, reliable and rapid regeneration protocols. These studies may thus be considered as a remarkable step towards further advanced research in the sense that application of biotechniques in a plant system requires a complete and quick plant regeneration system.

For all the *in vitro* experiments performed on both the crops, the explants (starting material) were taken from two weeks old aseptically grown seedlings on paper bridges with distilled water or half strength MS (Murashige and Skoog, 1962) medium [Plate 1; Fig. A-D]. MS medium supplemented with hormones was invariably used for maintaining all the cultures. The aseptic manipulations were conducted in the laminar air flow bench pre-sterilized with ultraviolet light for forty minutes. The cultures after inoculation were kept in a growth chamber under controlled conditions of light and humidity.

Outline, observation and results of the undertaken experiments are narrated below:
Micropropagation (production of plantlets *in vitro*) has emerged from theoritical confines to establish itself as a multidollar industry. The intent of micropropagation is an increased production of plants that are uniform and predictable of selected qualities. However, these procedures should strive for optima, with respect to certain parameters, namely the initial explant characteristics, nutrient formulation and culture environment.

Micropropagation is usually achieved in three steps

(1) Establishment of explant
(2) It's multiplication and
(3) Then it's rooting, which is a lengthy and time consuming process.

However, in cumin as well as ajowain, short and quick system for complete plant regeneration has been established using shoot tip as explant.

In case of *C. cyminum* production of regenerants via apical bud culture proved quite feasible. Main advantage of culturing these buds over any other mode of plantlet production lies in the fact that they harbour preexisting meristem. Hence these could be triggered easily for shoot bud proliferation. The experiment initiated with the inoculation of shoot tips on IAA containing MS basal medium. Although, multiple shoot induction could not be elicited, however, inherent meristematic property of the explant was considerably enhanced. Thus, an increase in length was observed by the first week of inoculation, with the emergence of new shoots from the meristematic cells present in the explant [Plate 2; Fig. A]. By the second week the shoot development was vigorous. With the development of shoots, rooting also concurred just simultaneously proportionate to the shoots above [Plate 2; Fig. B], by the mid second or third week. Full length of the plant, that is, with the well developed root and shoot system was observed by the forth week.

As hardening and acclimatization is the crucial necessity of *in vitro* regenerants, these plantlets were subjected to hardening process. They were then taken for soil survival experiments [Plate 2; Fig. C]. These plants were able to reach maturation and even flowered, giving a successful demonstration of tissue culture technique within time period of 35-45 days.

The result thus proved very significant in successfully obtaining regeneration in just one step and their further survival in soil made the micropropagation successful.

In *T. ammi* regenerants were obtained via shoot tips inoculated on MS medium supplemented with auxin IBA [Plate 3; Fig. A]. Further increase in the shoot length as well as emergence of new shoots were obtained on the same medium. The shoots grew vigrously and by the second week development of root system was also attained. Thus, complete plantlets with well differentiated root and shoot system were ready for transfer to the pots within three weeks time period [Plate 3; Fig. B]. The regenerated plantlets after hardening were transferred to the field [Plate 3; Fig. C].

These experiments thus established a model system for quicker plant regeneration on auxin alone in a single step, eliminating the requisite of a separate rooting medium in the candidate crops.

Efforts were also directed towards multiple shoot production from the shoot tip explant in case of *T. ammi*. However, the shoot tip explants for these particular set of experiments were excised from seedlings raised on NAA-BAP containing MS media. These explants yielded better results as compared to those excised from seedlings raised on paper bridges or on phytohormone devoid synthetic media.

These shoot apices when inoculated on MS medium, containing BAP in conjugation with IAA, proliferated into a large number (12-16) of multiple shoots within one week of inoculation [Plate 4; Fig. A]. After three weeks of incubation the shoots were separated individually and cultured on IBA containing medium [Plate 4; Fig. B]. This nutritional constitution initiated rooting, at the same time inducing a few more new shoots, thus establishing a protocol for repetitive multiple shoot production.

Moreover, in order to improve the above protocol for multiple shoot prodcution suspension culture was also tried using the shoot tips from seedlings raised on NAA-BAP containing medium. Most significant results were obtained when the shoot apices were cultured in agitated liquid MS medium containing very high levels of auxin IAA in conjugation with very low concentrations of BAP (less than 1.0 mg/l) [Plate 4; Fig. C], where in, the shoot tips proliferated to give rise to multiple shoots (6-10) within a week. The shoots grew vigrously to their complete length within 10-15 days. The shoots for their further elongation and rooting were then separated individually and transferred to solidified MS medium incorporated with IAA or IBA singly in the full strength MS medium [Plate 4; Fig. E].

Thus, complete plantlets were obtained just within 25-35 days of culture and could survive in the field conditions [Plate 4; Fig. D; Plate 5; Fig. A-B].

Hence, tissue culture has been affirmed as a means to cultivate plant parts whether a single cell, tissue or an organ under aseptic conditions to overcome many problems of conventional agricultural practices. Moreover, tissue culture system also appears as an ideal system which helps in investigating physiological biochemical and genetical structural problems related to plants.

Thus, studies with plant cell cultures clearly have bearing upon a variety of problems still unsolved at the grass root level as well as for their applied research. In the application of *in vitro* methods for the improvement of the genetic potential of plants, for their oil content and medicinal values, the regeneration of plant attains special significance. Although totipotency is probably characteristic of all plant cells, but it's expression may be limited to particular cells. It is apparently confined to cells that are identified as meristemoids. Meristemoids are often located in specific tissues and organs. Murashige and Nakano, way back in 1967 also reported that tissues excised from the more recently produced parts of a herbaceous plant are more regenerative than those from older regions. Higher regenerative ability of meristematic cells has also been reported by Gamborg *et al.*, 1974 as well as Mukhopadhya and Bhojwani, 1978. This implies to the fact that perhaps the most important determinant of plant multiplication and quality of regenerated plants is the initial explant. In the absence of a suitable explant success is at best limited. Murashige, 1974 also mentioned plant regeneration accomplishment from various explants *viz.*, leaves, stem, cotyledons, microsporophytes, as well as shoot tips. Reports are also available which favour use of diverse explants such as hypocotyl (Meiners *et al.*, 1991; Schroeder and Stimart, 1997; Audichya, 1999), cotyledon (Knittel *et al.*, 1991; Chraibi *et al.*, 1992; Sharma, 1996), cotyledonary node, (Meiners *et al.*, 1991; Distabanjong and Geneve, 1996; Sharma, 1999), nodal segment (Lakshmi Sita, 1986; Hussein, 1997) in various plant species.

However, during our experimentation with the candidate crops, shoot tips emerged as the most amenable explant as also supported by Luckner *et al.*, 1984; John and Batra, 1994; Sardana, 1998.

The next determinant for plant regeneration is providing optimum provisions of nutrient formulation.Studies have been conducted on the nutritional factors by Singha *et al.*, 1987.

According to Skoog and Miller, 1959 the relationship of auxin – cytokinin balance to root and shoot initiation remains at the seat of plant regeneration. The suitability of shoot tip explant for regeneration and it's sensitivity to various hormones is due to the activity of meristematic cells, which are actively dividing and are known to have dense cytoplasm with much more uniform and homogenous composition. Present studies on two medicinal plants revealed that when auxin used singly proved most effective.

However, Murashige (1974) developed the concept of developmental stages for micropropagation mentioning 3 stages *viz*:

Stage 1: Explant establishment
Stage 2: Multiplication of the propagules
Stage 3: Rooting and hardening for planting into soil. This concept stimulates the awareness that a single medium usually is not sufficient for *in vitro* plant multiplication and regeneration. Transferring the propagules through a series of specially designed chemical and physical environment holds the key to success.

Though, sometimes deviation from this above concept are also observed, like in the present work, where complete plant regeneration with shoot elongation and rooting was obtained on the same medium composition in both the crops. Herrera *et al.* (1990) for the first time reported similar kind of a technique in *Digitalis thapsi*, eliminating separate medium requisite for rooting.

Auxins are usually required for shoot growth, but in the present case both *Cumin* and *Trachyspermum*, IAA proved effective in inducing rhizogenesis. However, rhizogenic effect of IAA was contradicted by the report of Thimann (1977) who attributed the inhibitory effect of auxin on root elongation. Nevertheless, several reports (Jha *et al.*, 1983; Bajaj and Mahopatra, 1987; Gulati and Jaiwal 1990; Dave, 1994; Ajita, 1996) are available in support of the use of auxins, particularly IAA.

In case of ajowain multiple shoot production was also accomplished from the shoot tip explants cultivated on cytokinin (BAP) containing nutrient medium. Reports on *Brassica* (George and Rao, 1980); *Carthamus* (George and Rao, 1982); *Sesamum indicum* (George *et al.*, 1989; Gogna, 1993); *Syzygium aromaticum* (Mathew and Hariharan, 1990) also stand in consonance to this result.

Enhanced multiple shoot production in *Trachyspermum* was obtained during the experiments by germinating the seedlings *i.e.* the source of explant on MS medium incorporated with an auxin and cytokinin (NAA-BAP). George and coworkers in 1987 also reported presoaking of *Sesamum indicum* seeds and their subsequent germination on cytokinin containing media. Jain and Datta (1992) also studied the effect of presoaking the explants on organogenesis.

Thus, present investigations reflect upon shoot multiplication facilitated by exogenous cytokinins with low concentration of auxin indicating presence of endogenous auxins in the shoot tip explants. This indicates that shoot tip being the principal site for auxin biosynthesis, which may produce multiple shoots where cultured on cytokinin alone.
Another remarkable aspect observed was the effect of suspension medium on multiple shoot production.

Miller and Murashige (1976) mentioned that choice between gelled and liquid formulations should not be made arbitarily as it may affect the plant growth, multiplication as well as survival. This view holds true in the present plant systems, where the complete system of shoot multiplication in *Trachyspermum* was accelerated by one week. Snir and Erez, 1980 also reported faster growth rate of apple shoot tips on liquid medium. The use of liquid medium for *in vitro* culture has been considered an ideal technique for mass production as it reduces manual labour and facilitates change of medium. Consequently this technique has been employed by Teisson and Alvard (1995) on a number of species *viz.* Coffee, *Heavea*, *Musa*.

In all the cases root induction was obtained on sole auxin containing media.

Role of auxin for rooting has also been supported by the reports of Lakshmanan and Dhanalakshmi, 1990; Gogna, 1993; Rout *et al.*, 1997; Bhuchar *et al.*, 1999. When well developed shoots are produced, they are separated and cultured for rooting.

However, the plantlets during their *in vitro* cultivation grow under special climatic and light conditions, making them very sensitive. Thus arises the need for their hardening and acclimatization for their adaptation to an environmental change. Keeping this in view, during the present investigation *in vitro* produced regenerants were maintained under high humidity conditions for initial two weeks with their growing in a mixture of soil and vermiculite. These are then gradually exposed to the natural climatic conditions. Similar hardening process has been used by Kaul, 1987; Batra, 1998; Sharma, 1999.

Thus, to conclude, plant cells remain an important source of medicinal compounds and account for over a quarter of all prescribed drugs with an annual market value of over $ 3 billion (Venkataraman, 1998). In view of this, as well as the growing awareness about the side effects of the synthetic drugs, has provided the necessary impetus to hasten the pace of research in medicinal plant biotechnology.

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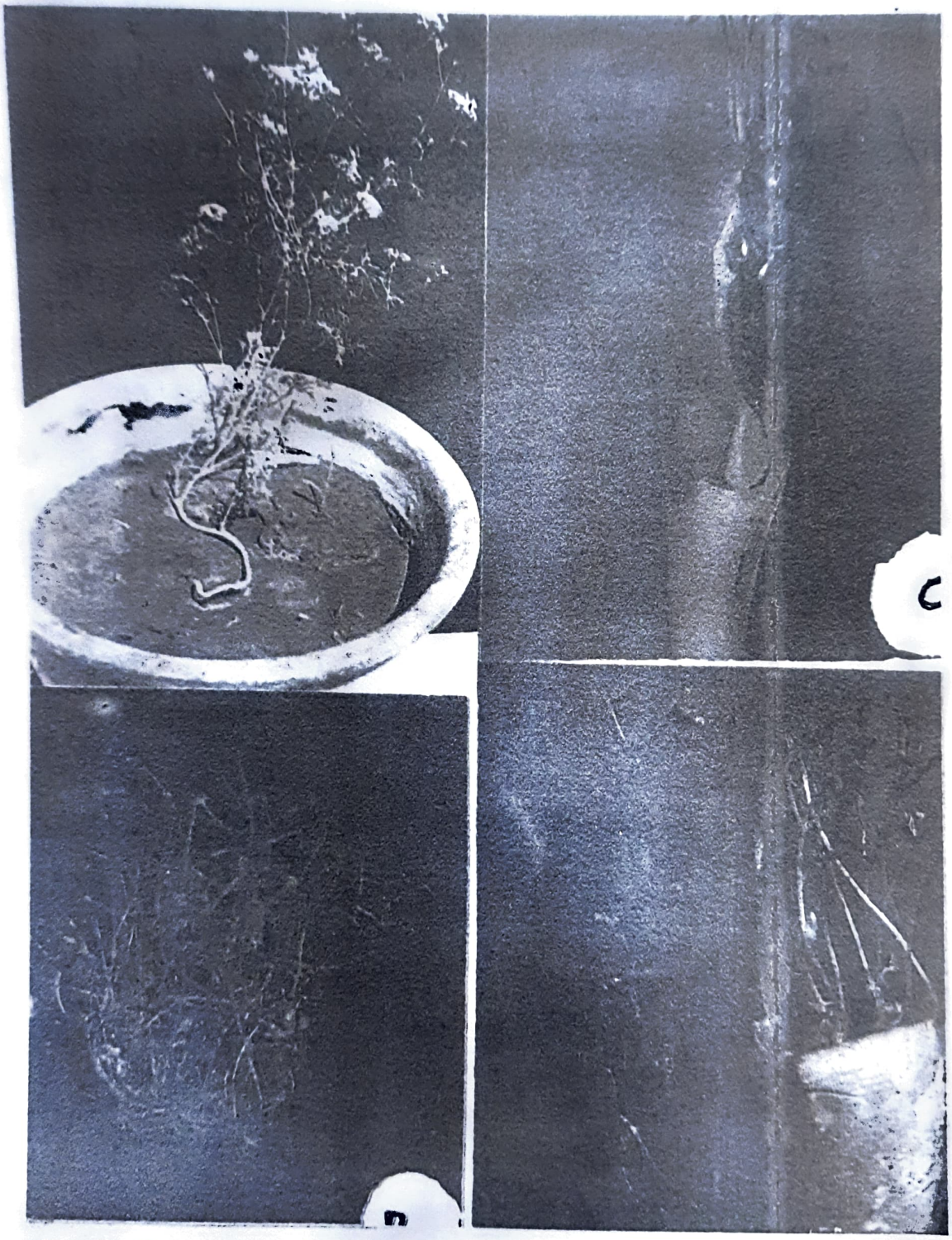


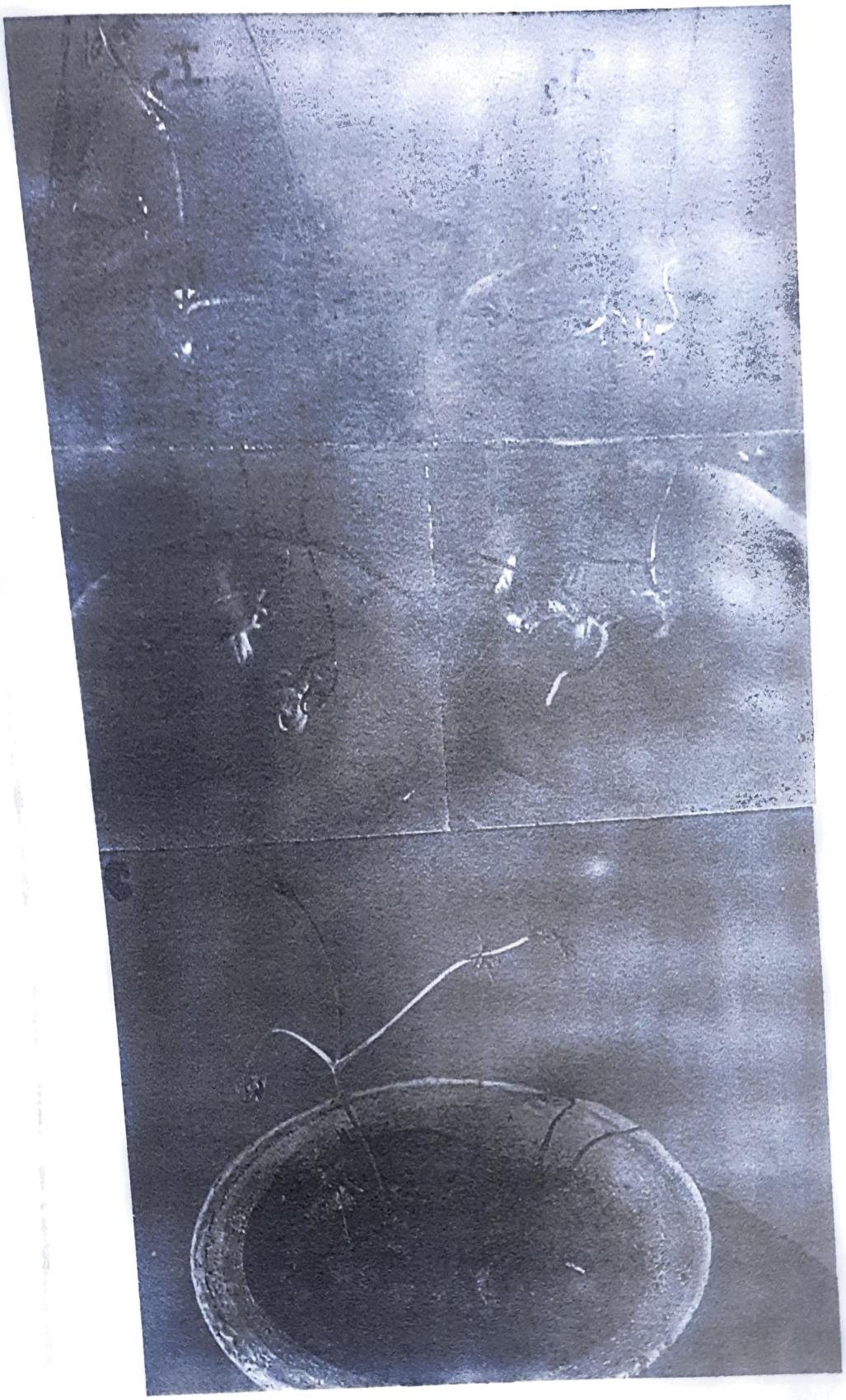
Aseptically grown seedlings of *Trachyspermum ammi* and *Cuminum cyminum*

Fig. A Germinated seeds of Cuminum cyminum

Fig. B Germinated seeds of *Trachyspermum ammi*

Fig. C *In vitro* germinated seeds of Cuminum cyminum on paper bridge.

Fig. D *In vitro* germinated seeds of *Trachyspermum ammi* on paper bridge

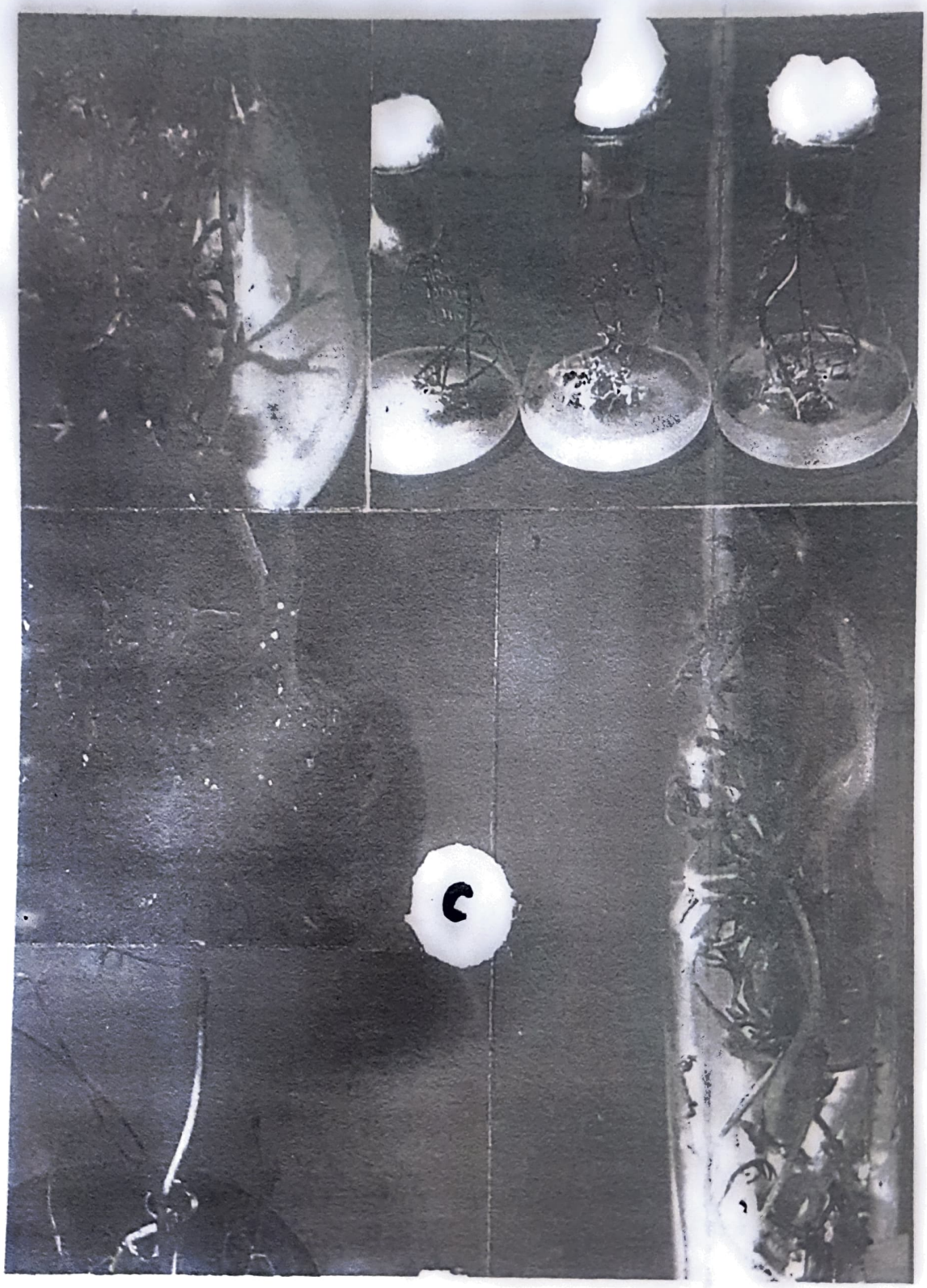


Shoot tip culture leading to complete plant regeneration in *Trachyspermum ammi*.

Fig. A *T. ammi* regenerants on MS medium + IBA

Fig. B Root initiation

Fig. C Pot transfer



Rapid shoot multiplication in T. amnni.

Fig. A Multiple shoot proliferation

Fig. B Transfer of separated shoots on IBA

Fig. C Multiple shoot proliferation in liquid medium containing BAP and IAA

Fig. D Separated shoots transferred to solid media for rooting

Fig. E Plantlet transferred to soil

Fig. A Acclimatization of *in vitro* regenerated plantlets of *T. ammi.*
Fig. B Pot transfer

Shoot tip culture leading to complete plant regeneration in Cuminum cyminum in one step.

Fig. A Growth of explant after inoculation
Fig. B Root induction
Fig. C Complete plantlets transferred to soil