AIM OF THE PRESENT WORK

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Plant tissue culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. Biotechnological applications of plant tissue cultures represent the most updated reviews on current techniques in plant culture in the field. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large scale culture of plant cells from which these metabolites can be extracted. In addition to its importance in the discovery of new medicines, plant cell culture technology plays an even more significant role in solving world hunger by developing agricultural crops that provide both higher yield and more resistance to pathogens and adverse environmental and climatic conditions [73].

Therefore, the initiation of of a successful tissue culture for two important medicinal plants (*O. basilicum* L. and *P. anisum* L.) and investigation of new biological activities of the produced secondary metabolites from tissue cultures is the top of our crucial research objectives.

From the previous literature, the thesis –in hand- is rationalized to and concerned with the following standpoints:

1. The proper conditions that allow the callus to produce the essential oils of medicinal interest.
2. The pattern of production of essential oils according to the time course.
3. Examination of the pattern of induced cytodifferentiation and organogenesis during culture time.
4. Identification of components of the produced essential oils and their relative percentage.
5. Biological screening of the produced essential oils for their cytotoxic, antioxidant, and immunostimulant activities.

Part I

Plant tissue culture of *Ocimum basilicum* L. and *Pimpinella anisum* L. and examination of calli for cytodifferentiation and organogenesis.

INTRODUCTION

Plant tissue culture

Plant tissue culture, the growth of plant cells outside the intact plant; it is an experimental technique through which a mass of cells is produced from tissue explants. It relies on maintaining plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells (callus) for an extended period of time or regenerated into whole plants. The callus produced can be utilized directly to regenerate plantlets or to extract or to manipulate some primary and secondary metabolites. Callus culture and suspension culture are the basic technique used to produce the desired metabolites of plants. Because plant cell culture is not affected by changes in environmental conditions, improved production may be available in any place or season. Therefore, studies on the production of useful metabolites by plant cell culture have been carried out on an increasing scale since the end of the 1950's. Their results stimulated more recent studies on the industrial application of this technology in many countries [74].

Organogenesis

Although observations were made on the formation of organs *in vitro* since the early studies in plant tissue cultures, there is still paucity of factual information on the induction of organogenesis in isolated and cultured plant tissues. In a given callus only few cells are involved in the initiation process, and the onset of the process is asynchronous and somewhat unpredictable [75].

The underlying factors involved in organogenesis are obscure because the stimuli may involve components of the medium, endogenous compounds produced by the culture, and substances carried over from original explants [76]. With prolonged subculture of callus several changes may occur in the system. These include hormone habituation, the loss of organogenic potential, and changes in the external characteristics of the callus such as friability [77] .

The first indication that *in vitro* organogenesis could be chemically regulated was given by Skooge [78] who had studies led to the hypothesis that organogenesis is controlled by a balance between cytokinin and auxin. Probably the most precise regulation of organ formation has been achieved with epidermal and subepidermal explants consisting of few cell layers in thickness [79].

The formation of floral buds, vegetative buds, and roots has been demonstrated in thin layer explants by several species by regulating auxin:cytokinin ratio, carbohydrate supply, and environmental conditions [80,81]. Certain isolated tissue layers, in species that readily regenerate organs *in vivo*, showed a remarkable potential to form organs during culture [75].

An advanced hypothesis demonstrated that organogenesis in callus starts with the formation of clusters of meristematic cells capable of responding to factors within the system to produce a primordium [81]. Depending on the nature of internal factors, the stimuli can initiate root, shoot, or an embryoid. Many observations on organ formation in cultured tissues support the hypothesis that localized meristematic activity precedes the organized development of roots and shoots [82] .

The factors that regulate the origin of these meristematic zones are not understood. Since these zones are located in the vicinity of the tissue –medium interface, it has been suggested that physiological gradients of substances diffusing from the medium into the tissue may play a role in determining the loci at which meristemoids are formed [83]. The merstimatic region may also act as a sink and withdraw essential metabolites from the surroundings cells, thus localizing the meristematic zone [84].

Root initiation

It is a type of organogenesis most frequently found in cultured tissues. Root initiation frequently occurs after the cultured tissue has produced buds, and shoots development undoubtedly alters the endogenous hormones within the culture [85] .

The controlling factors involved in root formation include mineral salts, sugar, auxin, temperature, and light [86]. In some cultured tissues auxin promotes root formation, whereas in other systems, exogenous auxin is inhibitory, and rhizogenesis can be stimulated by antiauxin[87] . Such reports are not contradictory because the endogenous cytokinin:auxin balance is the key factor in the initiation of the process [77].

The potential to form roots may decline after several subcultures, but the reason for this loss of morphogenetic potential is unknown [75]. It may be due to exhaustion of a specific factor present in the initial explants and no longer synthesized *in vitro* [88]. Another possible explanation for the loss of this characteristic is that epigenetic changes involved selective gene expression have occurred in the cultured tissues [89] .

The appropriate culture conditions for root formation in some species or variety may be completely ineffective in a closely related organism [83].

Several investigators have evidence of phenolic compounds acting in combination with auxin to promote organogenesis [77]. The combination of phloroglucinol and indolebutyric acid, for example, was much more effective than auxin alone in stimulating rooting [90]. Apparently there is no relationship between ploidy changes and rhizogenesis [91].

Shoot initiation

Also called caulogenesis, in cultured plant tissues can be induced in many systems by an appropriate balance of exogenous auxin and cytokinin, and in some cases, either one or the other of these growth regulators must be omitted from the medium in order to produce buds [83].

Buds are initiated in many dicot callus cultures by transferring the callus to a medium containing a cytokinin: auxin ratio in the range of 10 to 100, whereas callus production is favored by an auxin: cytokinin ratio of 10 to 100. The term “reversal transfer” has been applied to the transfer of a culture from a callus inducing medium to a shoot inducing medium [84,92].

In many monocot cultures exogenous cytokinin may be unnecessary for the initiation of buds [75]. The omission of auxin from the medium may be sufficient to induce shoot formation in these cultures, and two successive transfers on auxin-free media are recommended [85].

In addition to cytokinins, a variety of related compounds are capable of replacing cytokinins for shoot induction. These chemicals include substituted purines, pyrimidines, and ureas [77]. In addition, adenine sulfate is capable of functioning as a cytokinin in the induction of buds [93,94].

Auxin: cytokinin supplements or omissions from the medium have failed to induce shoots in the culture of many species and Street[83] has offered many reasons for these failures:(1) additional hormones may be required; (2) endogenous hormones may accumulate, and their inhibitory effect on organogenesis is not reversed by the exogenous hormones; and (3) cultural conditions involving nutritional and physical factors may block the onset of the process.

The relative concentration and spectrum of endogenous gibberellins like compounds change during shoot initiation, and this group of hormones plays a role in organogenesis [77]. Gibberellins, in general tend to suppress shoot and root formation. Shoot-forming callus accumulates starch, and this biosynthetic process is thought to be required for bud initiation. The inhibitory effect of GA3 on caulogenesis has been related to the lowering of starch content of bud-forming cells [84,95].

Endogenous ethylene may be a factor in caulogenesis. Early in the culture this gaseous hormone blocks organogenesis, but during primordial formation ethylene enhances their development [96].

Carbohydrate metabolism is another factor to be considered in caulogenesis. In addition to serving as a respiratory energy source, the exogenous carbohydrate may act as osmotic agent. Mild alterations that are reflected osmotic stress apparently causes biochemical alterations that are reflected in callus growth and morphology [97].

Xylogenesis (Induction of the differentiation of xylem cells)

Xylogenesis is a type of cytodifferentiation which refers to a morphological and biochemical specialization of a given cell, as well as the developmental process leading to this unique condition. In the latter sense, differentiation has been defined as the summation of processes leading to the acquisition or loss of specific metabolic components that serve to distinguish daughter cells from each other and from the parental cell [98].

The basic problem of cytodifferentiation centers on the genetic programming of a cell in order to produce these metabolic changes. Determination refers to the process by which a cell becomes restricted to a new pathway of specialization, the transient period during which the new cytodifferentiation is initiated is called the point of determination [99].

Xylogenesis as a type of cytodifferentiation, can be easily studied for several reasons (1) with the exception of sieve elements, few other cell types can be experimentally induced to form under conditions of plant tissue culture; (2) xylem cells can be easily identified under light microscope because of unusual pattern of secondary-wall thickening; (3) cytodifferentiation occurs at a relatively rapid and predictable rate [100]; (4) the chemical requirements for induction of xylogenesis are relatively simple.

Although a typical culture medium is usually employed, the minimal chemical requirements for xylogenesis in excised parenchyma tissue are an auxin, a cytokinin, and a carbohydrate. Some investigations support the view that ethylene is a hormonal requirement [101,102]. Gibberellic acid may interact with other hormones during the *in vitro* differentiation of secondary xylem fibers [103].

The developmental process, from the point of determination to the fully differentiated xylem cell, has been termed "cytodifferentiation sequence". Xylem cells formed under *in vitro* conditions exhibit the following stages of development after determination: cell enlargement, secondary wall deposition and lignifications, wall lysis and cell autolysis [99]. Cells differentiated in tissue cultures include vessel elements or wound vessel members, tracheids, and cells that resemble xylem fibers [104].

Xylogenesis has been studied in two types of callus: wound callus still attached to the original primary explants and explants derived from a stock that has experienced numerous subcultures and is devoid of any parental tissue [105]. The pattern of xylogenesis varies considerably from one culture to another and presumably is governed by several factors.

For example, xylem formation in explants of lettuce pith depends, to some extent, on the combination of auxin and cytokinin present in the medium [106]. In callus cultures vascular nodules consisting of xylem, phloem, and a cambial zone may be present. The arrangement and cellular composition of vascular nodules can be altered by varying the relative amounts of auxin and sucrose in the medium [107-110].

Studies have shown that tracheary element differentiation is closely related to cell cycling apparently activity, although some cells apparently differentiate directly without any obvious expression of mitotic division [75].

Genus *Ocimum* tissue cultures

In vitro rosmarinic acid accumulation in *O. basilicum* was reported. Leaf derived suspension cultures of*O. basilicum* were cultured on a media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) 2 mg/l and Naphtylacetic acid (NAA) 2 mg/l as growth regulators accumulated rosmarinic acid up to 10 mg /g dry wt. a value up to11times more than callus culture or in leaves of donor plants [111].

Propagation and conservation of four pharmaceutically important herbs, *O. Americanum* L. (horny basil), *O. Basilicum* L. (sweet basil),*O. Gratissimum* (shrubby basil), and *O. Santcum* L. (sacred basil) was attempted using synthetic seed technology. Synthetic seeds were produced by encapsulating axillary vegetative buds harvested from garden-grown plants of these four *Ocimum* species in calcium alginate gel. The gel contained Murashige and Skoog (MS) nutrients and 1.1-4.4 µM benzyladenine (BA). The highest frequency shoot emergence and maximum number of shoots per bud were recorded on media containing BA. Both shoot and root emergence from the encapsulated buds in a single step was recorded on growth regulator-free MS medium as well as on vermi-compost moistened with half-strength MS medium. Rooted shoots were retrieved from the encapsulated buds of *O.*

Americanum, O. Basilicum and *O. Santcum* on these two media, whereas shoots of *O. Gratissimum* failed to root, the encapsulated buds could be stored for 60 d at 4°C. Plants retrieved from the encapsulated buds were hardened off and established in soil [112].

An efficient plant regeneration protocol was successfully developed for *O. basilicum* . Explants from 4 wks.old, seedlings yielded the highest frequency of 85% regeneration with an average of 5.1 shoots per explant. The regeneration protocol was performed on three basil varieties (Sweet Dani, methylcinnamate, Green Purple Ruffles). Callus and shoot induction was initiated on MS basal medium supplemented with16.8µM of thidiazuron (TDZ) for approximately 30 d. Shoot induction and development were achieved by refreshing the induction medium after 14 d. The most morphogenetically responsive explants were from the first fully expanded true leaves of greenhouse-grown basil seedlings. All developing bud tissue demonstrated temporary anthocyanin expression. However, anthocyanin expression in Green Purple Ruffles remained stable until maturity. Developing shoots were rooted in the dark on media deprived of TDZ. Within 20 d, rooted plantlets were transferred and acclimatized under greenhouse conditions where they developed normal morphological characteristics [113].

An efficient protocol has been developed for rapid micropropagation of *O. basilicum*. Multiple shoots were induced by culturing shoot tip explants excised from mature plants on a liquid MS medium supplemented with 5 - 100 µM of TDZ for different treatment duration (4, 8, 12 and 16 d). The optimal level of TDZ supplementation to the culture medium was 50 µM for 8 d induction period followed by subculturing in MS medium devoid of TDZ as it produced maximum regeneration frequency (78 %), mean number of shoots (11.6 ± 1.16) and shoot length (4.8 ± 0.43 cm) per explant. A culture period longer than 8 d with TDZ resulted in the formation of fasciated or distorted shoots. The regenerated shoots rooted best on MS medium containing 1.0 µM indole-3-butyric acid (IBA). The micropropagated shoots with well developed roots were established in pots containing garden soil and grown in greenhouse with 95 % survival rate. The regenerated plants were morphologically uniform and with similar growth characteristics to the donor plants [114].

A method for micropropagation of *O. basilicum* using nodal explants was reported. Axillary shoot bud proliferation was initiated from nodal explants cultured on MS medium supplemented with various concentration of BA (0.25-2.0 mg/l) and kinetin (KN) (0.25-2.0 mg/l). Maximum numbers of shoots (6.2±0.1) with average length (3.7±0.0) were induced on medium containing 0.5 mg/l BA. Shoots culture was established by repeated subculturing the original nodal explant on the same medium. Rooting of shoots was achieved on half strength MS medium supplemented with 1.0 mg/l NAA. Well-developed complete plantlets were transferred to plastic pots containing a mixture of (1:1) soil and vermiculite showed 90% survival rate [115].

Stereoselective oxidation of racemic1-arylethanols by cultured cells of *O. basilicum cv. Purpurascens* was reported. The biotransformation of racemic 1-phenylethanol (30mg) with plant cultured cells of (*O. basilicum cv. Purpurascens*, 5g wet wt) by shaking 120 rpm at 25C for 7 days in the dark produced (R)-(+)-1-phenylethanol and acetophenone in 34% and 24% yield, respectively. The biotransformation can be applied to other 1-arylethanols and basil cells oxidized the (S)-alchols to the corresponding ketones remaining the (R)-alchols [116] .

An effective protocol for complete plant regeneration via somatic embryogenesis has been developed for *O. basilicum* Callus was initiated from leaf explant of young plant supplemented with acid (2,4-D) 1.0 mg/l, 3% sucrose and 0.9% agar. The calli showed differentiation of globular structure embryos when transferred to MS medium containing 2, 4-D 0.5 mg/l and 6-Benzylaminopurine (BAP) 1.0 mg/l. The maximum globular structure embryos were further enlarged and produced somatic embryos in MS basal medium supplemented with (BAP) 1.0 mg/l, (NAA)1.0 mg/l and (KN) 0.5 mg/l [117].

Untransformed normal roots of *O. basilicum* harbored the maximum titers (0.98% g fresh weight basis) of RA (rosmarinic acid) compared to leaves and shoots. Hairy root cultures of *O. basilicum* transformed with *Agrobacterium rhizogenes* (ATCC-15834) showed three-fold increase in growth and RA production compared to the untransformed normal roots. Upon elicitation with fungal cell wall elicitors (CWE) from *Phytophthora cinnamoni*, the production of RA was enhanced 2.67-fold compared with the untreated control. Roots were induced to exude RA by fungal in situ challenge with *Pythium ultimum*. Absence of RA in the root exudates of stressed root cultures proves that RA under normal circumstances is not exuded [118].

The influence of exogenous cytokinins (BAP) and (TDZ), and auxins (NAA or IBA) when added to the artificial nutritive medium, for modeling the *in vitro* propagation through direct somatic embryogenesis in two cultivars of *O. basilicum* L. var. *Marseille* and *O. basilicum* L. var. *Red Rubin*) was studied. Starting from different types of explants (leaf, cotyledon, epicotyl, hipocotyl and radicle), the best embryogenic response was recorded for experimental variant consisting in TDZ and IBA in quantities of 1 mg/l and 0.5mg/l respectively, and cotyledon type explants [119].

An efficient protocol for *in vitro* propagation of *O. basilicum* through axillary shoot proliferation from nodal explants was induced in nodal explants on (MS) containing (BA). The nodal explants required the presence of BA at a higher concentration(1.0 mg/l 4.4 µM) at the initial stage of bud break; however, further growth and proliferation required transfer to a medium containing BA at a relatively low concentration (0.25 mg/l, 1.1 µM). Gibberellic acid (GA3) at 0.4 mg/l (1.2µM) added to the medium along with BA (1.0 mg/l, 4.4 µM) markedly enhanced the frequency of bud break. The shoot clumps maintained on the proliferating medium for longer durations, developed inflorescences and flowered *in vitro*. The shoots formed *in vitro* were rooted on half-strength MS supplemented with 1.0 mg/l (5.0µM) IBA. Rooted plantlets were successfully acclimated in vermi-compost inside a growth chamber and eventually established in soil. All regenerated plants were morphologically identical to the donor plants [120].

In vitro multiplication of *O. gratissimum* L. through direct regeneration from nodal explants was developed. Single node explants were inoculated on basal MS medium containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of (BAP), (KN), indole-3-acetic acid (IAA) or (IBA) for direct plant regeneration. Maximum numbers of shoot (14.3±1.5) were observed on the medium containing 0.5 mg/l BAP and 0.25 mg/l IAA after four weeks of culture. Regenerated shoots were separated and rooted on same half strength MS medium supplemented with 0.5 mg/l of IAA alone for three weeks. Well-developed complete plantlets were transferred on to special made plastic cup containing soilrite. Acclimatized plantlets were successfully grown in garden soil [121].

The comparative scavenging efficiency of *in vitro* grown *O. tenuiflorum* for Copper and Lead using Atomic Absorption Spectroscopy was reported. The comparative concentrations of Copper and Lead scavenged by *in vitro* grown *O. tenuiflorum* were estimated with respect to its *in vivo* grown counterparts using Atomic Absorption Spectroscopy. *In vitro* plants were grown on 2.5 mg/l 2, 4-D in MS basal medium. *In vitro* grown *O. tenuiflorum* showed lesser amount of both these elements as compared to field grown plants. Due to lower amount of the elements in *in vitro* plant bodies, they claim that these plantlets in future could be able to extract more amounts of heavy metals, when grown in industrially contaminated soils on a wider scale, proving them better phytoremediates [122].

Furthermore, an efficient plant regeneration protocol has been developed from nodal explants of *O. kilimandscharicum* Guerke, Axillary shoot bud proliferation was initiated from nodal explants cultured on MS medium supplemented with various concentrations of (BA) (0.5-3.0 mg/l), (KN) (0.5-3.0 mg/l) and 2-isopentenyladenine (2-iP) (0.5-3.0 mg/l). The maximum number of shoots [6.09±0.05], with average length 3.83±0.11 cm, was achieved with medium containing 1.0 mg/l BA. Shoot culture was established by repeated subculturing of the original nodal explants on shoot multiplication medium after each harvest of newly formed shoots. In this way, 20-30 shoots were obtained from a single nodal explant after 5 months. Rooting of shoots was achieved on half-strength MS medium supplemented with 1.5 mg/l (IBA) and 2% sucrose. Well-developed plantlets transferred to plastic pots containing soil and vermiculite (1:1) showed 81.13% survival. The genetic fidelity of *in vitro*-raised field-grown plants to the donor plant was ascertained from random amplified polymorphic DNA [RAPD] markers [123].

P. anisum **L. tissue cultures**

Fatty acid β-oxidation and glyoxylate cycle enzyme activities of induced glyoxysomes from *P. anisum* suspension culture were studied. Homogenates of dedifferentiated *P. anisum* suspension cultures grown in B-5 medium with sucrose as source of carbon show all but 3 glyoxysomal enzyme activities: NAD-dependent oxidation of palmitoyl-CoA, isocitrate lyase, and malate synthase are lacking. Substitution of 20 mmol/l acetate for sucrose leads to the appearance of these enzyme activities. Only then glyoxysomes with a buoyant density of 1.23 kg/l in sucrose gradients are formed showing the enzyme activities for both β-oxidation of fatty acids and glyoxylate cycle. Quantitatively and qualitatively they resemble glyoxysomes isolated from endosperm of 4 d old anise seedlings [124].

Quantitation of gibberellins and the metabolism of ^{3}H labeled gibberellin A_1 during somatic embryogenesis in *P. anisum* cell cultures have been studied. Embryogenesis could be regulated by presence or absence of (2,4-D), in the medium (+2,4-D= no embryogenesis, -2,4-D= embryo differentiation and development). For quantitation dwarfice bioassay was used. Embryos harvested after 14 d of subculture in media deprived from 2, 4-D had low levels (0.2-0.3 µg per g dry weight) of polar GA (e.g. GA_1-like), but much (3-22 times) higher levels of less-polar GA ($GA_{4/7like}$); GA_1, GA_4 and GA_7 are native to these cultures. Conversely, the undifferentiated cells in a non-embryogenic strain, and proembryos of an embryogenic strain in media supplemented with 2,4-D showed very high levels of polar GA (2.9-4.4 µg/g), and somewhat reduced levels of less-polar GA. For metabolism, cultures of anise undergoing somatic embryo development metabolized [^{3}H] GA_1 very quickly, whereas proembryo cultures of *P. anisum* metabolized [^{3}H]GA_1 slowly. The major metabolites of [^{3}H] GA_1 in anise were tentatively identified as GA_8-glucoside (24%), GA_8 (15%), GA_1-glucoside (8%) and the $\Delta^{1(10)}$ GA_1-counterpart (2%) [125].

A new, naturally occurring cytokinin was isolated from a cell culture of *P. anisum*. Its structure was determined as 6-benzylaminopurineriboside by the use of gas chromatography and mass spectrometry. Quantitation of this new plant hormone was accomplished by single-ion-monitoring measurements [126].

A tissue culture of *P. anisum* was set up that selectively promoted the production of epoxy-pseudoisoeugenol-(2-methylbutyrate), termed EPB. This compound served as the final molecule of the biosynthetic pathway in all labelling experiments conducted. The putative precursors were labelled with ^{13}C or ^{14}C. Based on labelling experiments as well as on enzymic reactions in a cell homogenate a genaral biosynthetic pathway for EPB with the following sequence; l-phenylalanine, trans-cinnamic acid, p-coumaric acid, p-coumaric aldehyde, p-coumaric alcohol, and trans-anethol was proposed. The biosynthetic step leading from trans-anethol to pseudo-isoeugenol involves migration of the side chain during the introduction of the second OH-group in the molecule (NIH-shift). The final biosynthetic steps to form EPB must be acylation and epoxidation of the propenyl double bond of pseudoisoeugenol [127].

Somatic embryogenesis in *P. anisum* was investigated using root explants from two clonal lines in MS medium with 1 mg/l 2, 4-D and 3 % sucrose and supplemented with 100 mg/l of fish (cod or herring) protein hydrolysates in combination with 5.0 mM proline. Proline and both fish hydrolysates stimulated 2,4-D-induced somatic embryo formation in one clonal line, but only cod hydrolysates stimulated somatic embryo formation in the other clonal line. So, the combination of proline and fish hydrolysates was most effective in stimulating embryo formation [128].

Interaction between *P. anisum* and *Pseudomonas* offers a simple system to study the physiological and biochemical mechanisms underlying hyperhydricity in *P.anisum* tissue culture. The ability of *Pseudomonas spp.* originally isolated from an *in vitro* clonal line of oregano to prevent hyperhydricity in *P. anisum* shoot cultures was studied through observations of morphological adjustments and physiological variations in water, chlorophyll, and phenolic content. The *Pseudomonas* spp. elicited chlorophyll and phenolic production with substantial implications for enhancing the micro-propagation efficiency [129].

Phenolic content in differentiated tissue cultures of untransformed and *Agrobacterium*-transformed roots of *P. anisum* was compared. To investigate the role of differentiation of *P. anisum* tissue cultures on total phenolic and anethole contents, (BAP) and (TDZ) induced shoot cultures were generated from roots of the A-8 clonal line and its *Agrobacterium rhizogenes*-induced genetically transformed derivative JB-10. Embryogenic cultures were induced following 2, 4-D treatment. Root cultures were multiplied on hormone-free medium. The effect of proline on differentiation and phenolic synthesis was also investigated. GC/MS studies indicate that anethole was not produced in root or other differentiated cultures. The predominant phenolic metabolite, however, was an anethole precursor (EPB). Total phenolics and EPB contents were highest in root cultures, which also correlated with higher proline content. Embryo and shoot cultures had reduced phenolic level and EPB and proline contents. Antioxidant activity in all differentiating cultures was high on day 60 compared to that on day 30, and there was no significant difference between differentiating tissues. This indicated that antioxidant protection might be linked not only to phenolics but to other nonphenolic metabolites as well [130].

Induction of accumulation and degradation of the 18.4-kDa oleosin in a triacylglycerol-storing cell culture of *P. anisum* was achieved. Two closely related anise cell-culture lines, Pal5 and Pal9, differ considerably in growth rate, potential to form somatic embryoids, triacylglycerol (TAG) storage and pattern of lipid-body proteins. In contrast to the line Pal9, line Pal5 grows very fast mainly as single cells, exhibits a low potential for somatic embryogenesis and its TAG content is relatively low. Under defined culture conditions, the TAG content of the line Pal9 can be increased to approximately 70% of that of ripe anise seeds. In contrast, only fairly low levels of the 18.4-kDa oleosin in Pal5 were detected using immunodetection. Limited sucrose supply in the medium resulted in TAG degradation and the concomitant decrease in the amount of immunodetectible 18.4-kDa protein in the Pal9 cell culture. Treatment with sorbitol, or abscisic acid and sorbitol in combination, enhanced TAG contents and also the amount of immunostained 18.4-kDa protein in the cell culture Pal9, Whereas no effect was found on either TAG content or18.4-kDa protein in the cell-culture line Pal5. The results of this study indicate that the Pal9 cell culture provides valid model system for investigations of lipid storage and mobilization in higher plant cells [131].

Endogenous levels of cytokinin nucleotides in *P. anisum* cell culture were determined during proembryonal, as well as embryonal development. In both cultures the maximum level of isopentenyladenine nucleotides was found during the first four days of incubation which correlated with the beginning of logarithmic phase. The concentration of zeatin nucleotides remained constant at a very low level. This study indicates a major role of cytokinins in cell division, but not in embryo differentiation [132].

To study the effect of exogenous cytokinins on growth and somatic embryogenesis in *P. anisum* cells, a cell culture of anise was grown in the presence or absence of 2,4-D. Application of isopentenyladenine or isopentenyladenosine (4.10^{-8} to 4.10^{-7} M) to the proembryonic culture supplemented with 2,4-D yielded an increase of the cell density, in contrast to a proembryonic culture grown without exogenous application of cytokinins. Embryogenesis was induced by transferring the cells to a hormone-free medium. Embryo development was promoted by isopentenyladenine and isopentenyladenosine (5.10^{-8} to 5.10^{-7} M), higher concentrations (5.10^{-6}M) inhibited embryogenesis. The effect of cytokinins on embryogenesis was only promotive until the third day of culture [133] .

Isolation and quantitation of isopentenyladenosine in *P. anisum* cell culture by single-ion monitoring, radioimmunoassay and bioassay was reported. Using these three different techniques, isopentenyladenosine was identified and quantitated in *P. anisum* cell line growing on a medium without cytokinin. Quantitations by gas chromatography-mass spectrometry and radioimmunoassay correlated well, whereas the estimates by the bioassay gave considerably lower values [134].

Accumulation of essential oils by *Agrobacterium tumefaciens*-transformed shoot cultures of *P. anisum* was achieved. Axenic transformed shoot cultures of *P. anisum* were established following inoculation of plant stems with the nopaline strain T37 of *Agrobacterium tumefaciens*. Total essential oil accumulated by transformed shoot cultures grown under continuous light was found to be 18% lower (per unit fresh weight of tissue) than that produced by untransformed shoot cultures incubated under similar conditions, but more than 89% lower than the yield of oil from the intact plant. The relative amounts of the principal components of the essential oil of the transformed shoot cultures, namely geraniol, β-bisabolene, trans-pseudoisoeugenol-2-methylbutyrate and trans anethole, were similar to those present in the parent plant, but significantly different from those of the untransformed shoot culture [135].

A simple *in vitro* protocol has been developed for large scale multiplication of plants from various explants of *P. anisum*. High frequency of multiple shoot formation was achieved from callus cultures derived from shoot apices, root and stem explants, and also from seed-derived calli. Somatic embryogenesis was observed in callus cultures derived from seeds and shoot apices. Complete plants developed from these embryoids. Direct regeneration of plantlets from shoot apices was also observed. Root formation occurred in all the cultures. The requirement for exogenous auxin and cytokinin for differentiation was found to be varying in different tissues [136].

High frequency organogenesis was achieved in *P. anisum* using hypocotyls explants from *in vitro* germinated seedlings. Both cytokinins, BAP and KN induced shoot regeneration but the effect of BAP was more pronounced. High frequency shoot regeneration (45 shoots/ explant) was obtained on BAP 1 mg/l. Both cytokinins were also tested in combination with auxins NAA and IAA. Interaction of BAP and KN with NAA and IAA increased the length of regenerated shoots. The regenerated plants were normal and healthy [137].

A- Materials

1. Plant material

   P. anisum L. fruit were purchased from a local market in Alexandria. *O. basilicum* L. fresh leaves were continuously obtained from cultivated plant in the garden of Faculty of Pharmacy, University of Alexandria.

2. Chemicals
   - **i.** Murashige and Skoog' s medium (MS)
     (Caission Laboratory, USA)
   - **ii.** Growth regulators (plant hormones or phytohormones)
     Benzyladenine (BA)
     (Sigma Chemical Company, St Louis, Mo63178)
     2, 4-Dicholorophenoxyacetic acid (2, 4- D)
     (Eastman Kodak Chemical Company, Rochester, NY14650)
     Kinetin (KN)
     (Sigma Chemical Company,USA)
   - **iii.** Agar agar bacteriological grade (Bact agar agar type800, B&V, Parma, Italy)

   Miscellaneous
   - **iv.** Sodium Hypochlorite 5% solution (Chlorex)
   - **v.** Sterile distilled water
   - **vi.** Sucrose
   - **vii.** Chloral hydrate
   - **viii.** Phloroglucinol
   - **ix.** 0.1N Hydrochloric acid .
   - **x.** 0.1N Potassium Hydroxide.

3. Equipment
   - **i.** Laminar air flow cabinet.
     (NUAIRE, model no. NU-201-430E, series 20, year of MFG .2001)
   - **ii.** Multi-shaker (BIOSAN, PSU 20).
   - **iii.** Weighing technology apparatus (MonoBloc, PB303-S)
   - **iv.** Steam sterilizer (Rayapa, Model. AES-110)
   - **v.** Light microscopy (OpTIKA)

B. Method

1. Surface sterilization of both *O. basilicum* L. leaves and *P. anisum* L. seeds

a. Surface sterilization of *O. basilicum* L. leaves

Leaves were washed 3 times in tap water; surface sterilized in 20 %(v/v) hypochlorite solution for 20 min., rinsed 3 times with sterile water and blotted dry on sterile filter paper.

b. Surface sterilization of *P. anisum* L. seeds

P. anisum seeds were washed 3 times in tap water. Seeds were then surface sterilized in 20 % (v/v) hypochlorite solution for 25 min. and then rinsed 3 times with sterile water and blotted dry on a sterile mesh.

2. Establishment of static and suspension cultures

MS medium was used as the basal tissue culture nutrient. All media were adjusted to pH 6.0 prior to autoclaving at 121 °C and 15 psi for 20 min. Agar 1% (w/v) based MS medium with sucrose (30g/l) was supplemented with either 2,4- dicholorophenoxyacetic acid, 1.0 ppm (2,4-D, 1) with benzyladenine1.0 ppm, (BA, 1) or kinetin, 1.0 ppm (KN, 1). (BA, 1) was used to establish suspension cultures. The preparation of growth regulators stock solutions is shown in appendix I [page 98]

Agar based cultures are sometimes referred to as static cultures. Liquid cultures were established from static cultures by subculturing calli into the same media without agar. Static cultures were grown on 20ml media in 9 cm petri-dishes and liquid cultures in 50 ml Erlenmeyer flasks.

a. For *O. basilicum* L.

The leaves were scored on their dorsal side with a sterile scalpel blade and cut into pieces, about 1 cm^2 size. The explants were transferred to MS supplemented with (KN, 1) and (2, 4-D, 1) or with (BA, 1) and (2, 4-D, 1), 1% agar, 30g/l sucrose, PH adjusted at 5.6 and grown under continuous light (8000 Lux) at 22°C. The leaves formed yellowish-white callus [Figure3]; calli were transferred into the appropriate MS liquid media supplemented with (BA, 1) as growth regulator, 30g/l sucrose, PH adjusted at 5.6 and grown under continuous light (8000 Lux) at 23°C ± 1°C at 100 rpm and subcultured every 2 weeks.

b. For *P. anisum* L.

Seeds were cultured on basal MS medium in 9 cm petri dishes and left to germinate under continuous light (8000 Lux) at 22°C. Seeds began to germinate on approximately 8^{th} to10^{th}day [Figure5]. One month old hypocotyls aseptically were transferred to MS medium supplemented with (KN, 1) and (2, 4-D, 1) or with (BA, 1) and (2, 4-D, 1) and grown under continuous light (8000 Lux) at 22°C. Calli with grown aerial parts and sometimes roots and aerial parts were formed after approximately one month (organogeneasis). Static culture calli were transferred into the appropriate MS liquid media supplemented with (BA, 1) as growth regulator, 30g/l sucrose, PH adjusted at 5.6 and grown under continuous light (8000 Lux) at 23°C ±

1°C at 100 rpm and subcultured every 10 days.

Upon examination of formed calli using optical microscope, we observed that cytodifferentiation have occurred at many levels.

i. *O. basilicum* L.

By optical microscope we observed the formation of hairs which described as:

non-glandular, uniseriate, multicellular, covered with warty cuticle, and having a pointed end.

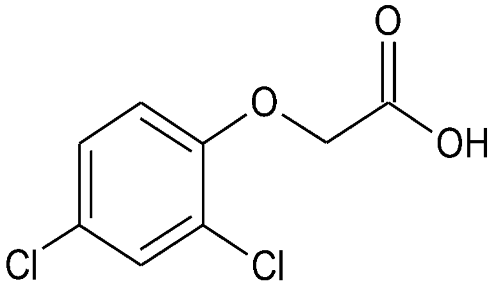
ii. *P. anisum* L.

Callus of anise differentiated into aerial parts (shoots and leaves) with high cytodifferentiation like formation of stomatal cells, hairs, and xylogenesis was also observed.

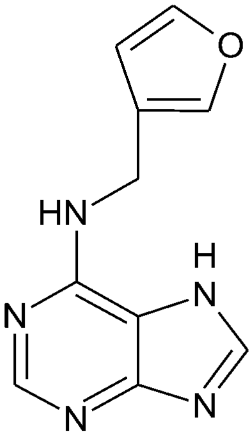
Chemical structure of the utilized plant growth regulators (phytohormones)

AUXINS

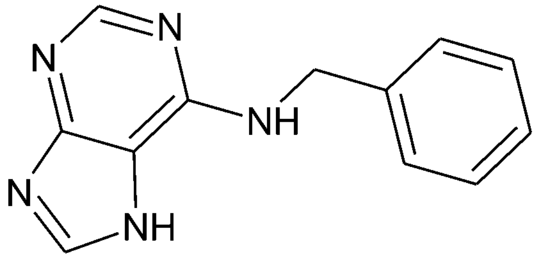
2, 4-Dichlorophenoxyacetic acid (2, 4- D)



CYTOKININS



Kinetin (KN)



Benzyladenine (BA)

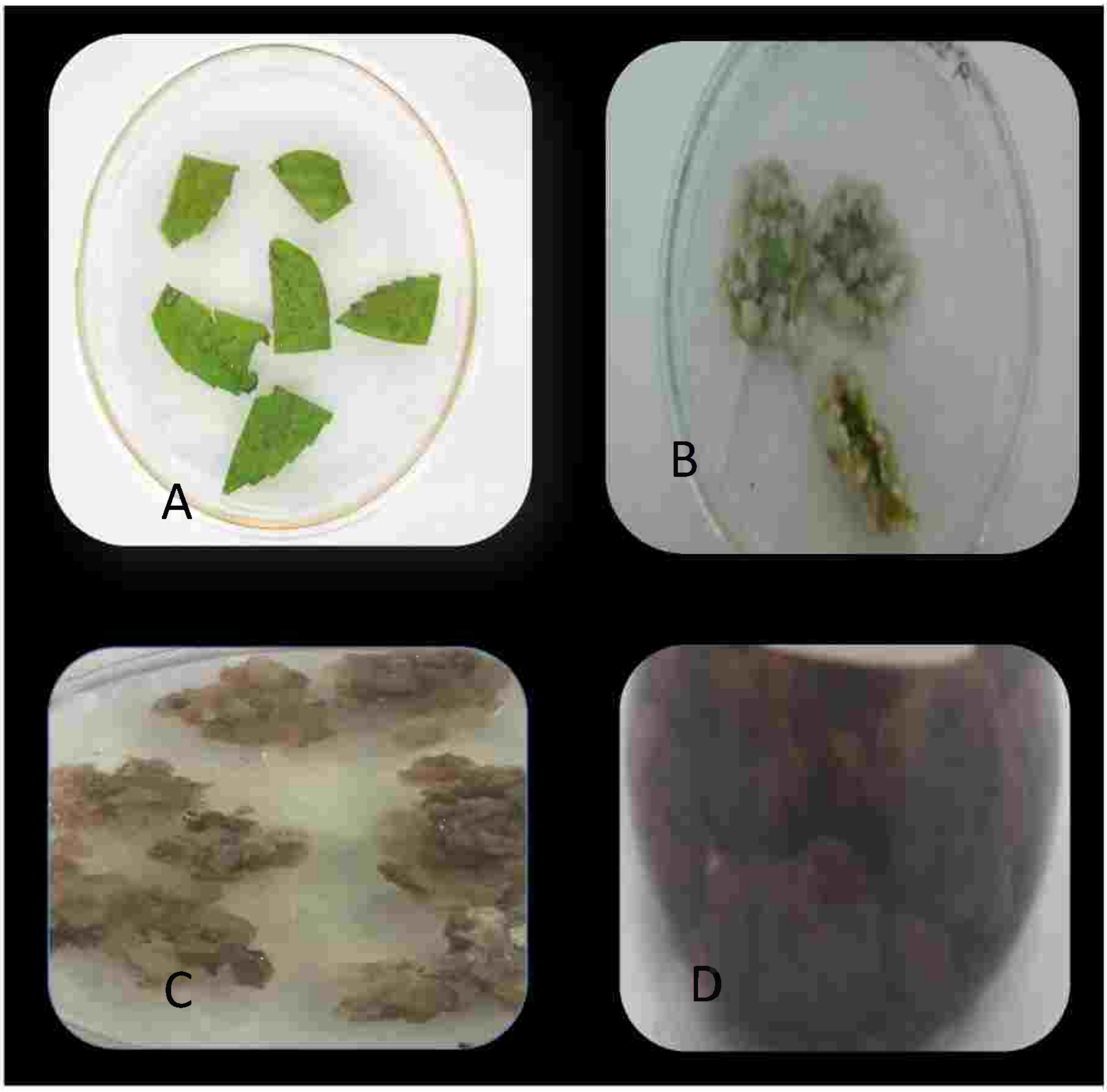


Figure 3. Formation of *O. basilicum* L. callus in a hormonal combination of 2, 4- D 1 and BA 1 static culture.

- **A.** Leaf explants of *O. basilicum* cultured on MS 2,4- D 1 and BA 1static medium.
- **B.** Yellowish white callus of *O. basilicum* after 2 weeks of the culture time.
- **C.** Brownish calli of *O. basilicum* after one month of the culture time.
- **D.** Suspension culture of *O. basilicum* calli containing MS BA 1liquid medium after 6 weeks of culture initiation.

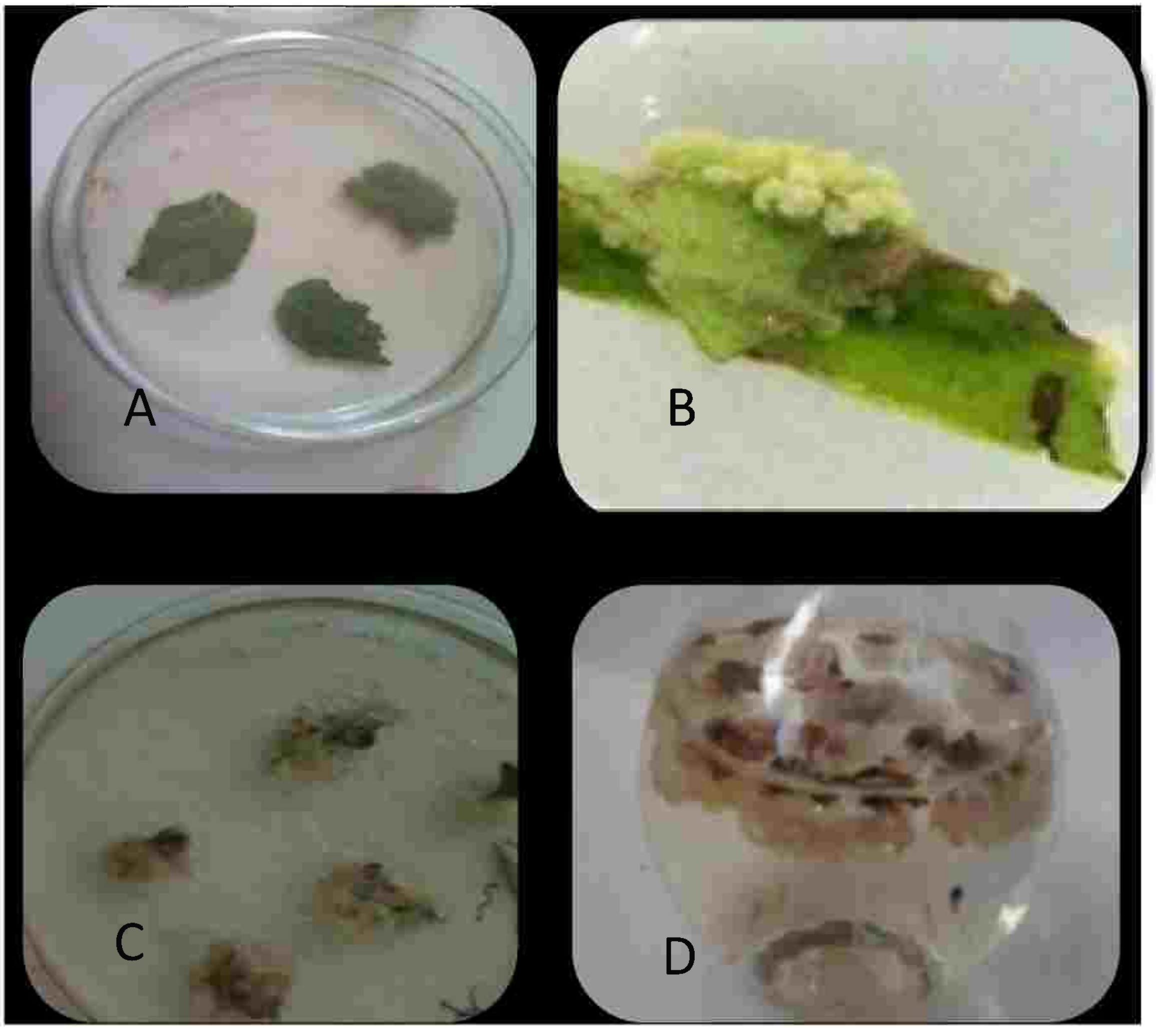


Figure 4. Formation of *O. basilicum* L. callus in a hormonal combination of 2, 4- D 1 and KN 1 static culture.

- **A.** Leaf explants of *O. basilicum* cultured on MS 2,4- D 1 and KN, 1static medium.
- **B.** Yellowish white callus of *O. basilicum* after 20 days of the culture time.
- **C.** Yellowish brown calli of *O. basilicum* after 6 weeks of the culture time.
- **D.** Suspension culture of *O. basilicum* calli containing MS BA 1liquid medium after 2 months of culture initiation.

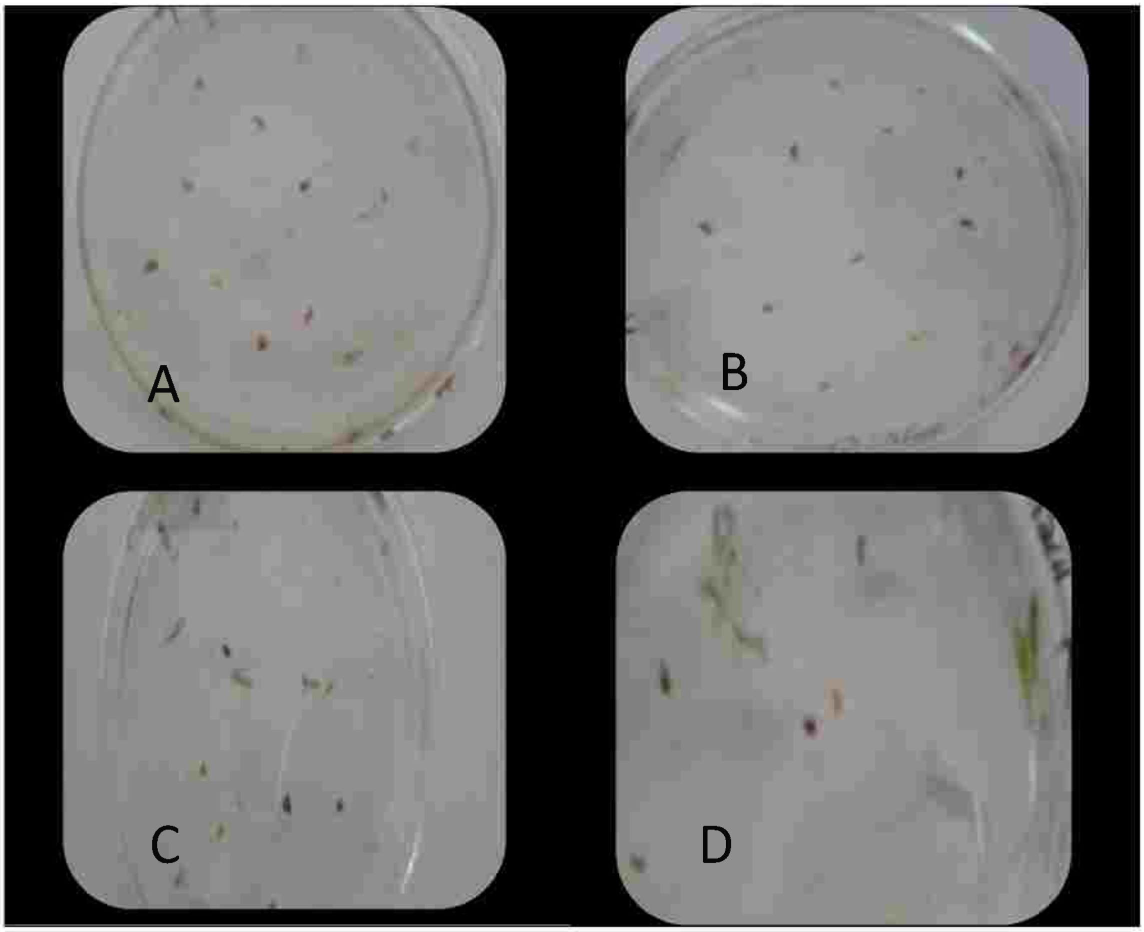


Figure 5. *P. anisum* L. seedlings on basal MS medium

- **A.** Seedlings of *P. anisum* at the first day.
- **B.** Seedlings of *P. anisum*, 10 days old.
- **C.** Seedlings of *P. anisum*, 20 days old.
- **D.** Seedlings of *P. anisum*, 1 month old.

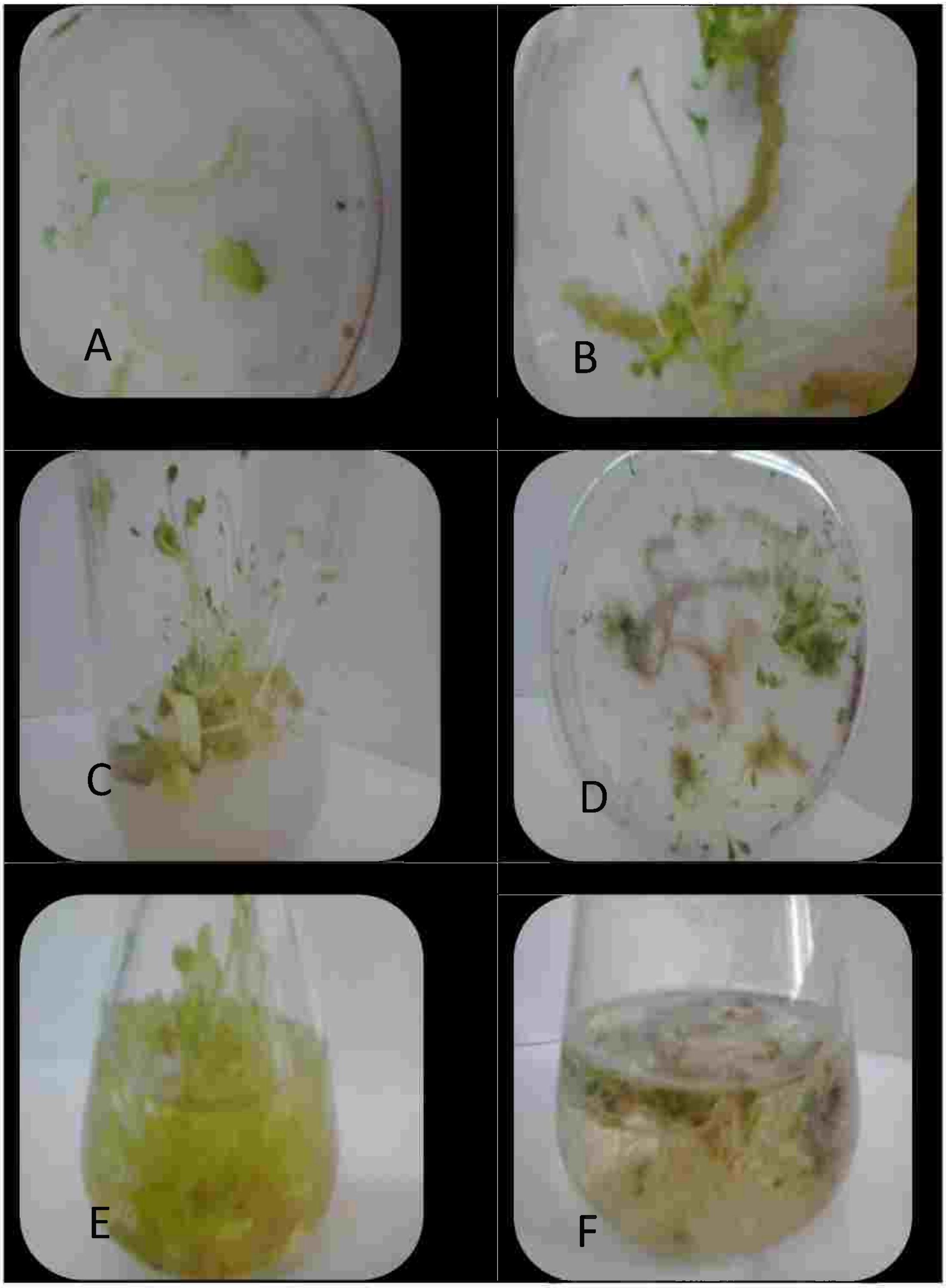


Figure 6. Formation of *P. anisum* L. callus in a hormonal combination of 2, 4- D 1 and BA 1 static culture.

- **A.** *P. anisum* callus with small shoots and leafy structure on MS 2,4- D, 1and BA, 1 static culture after 2 weeks.
- **B.** *P. anisum* callus with shoots and leafy structure on MS 2,4- D, 1and BA, 1 static culture after 20 days.

C. *P. anisum* callus with branched aerial parts on MS 2,4- D, 1and BA, 1 static culture after 1 month.
D. *P. anisum* callus with small shoots, leafy structure and roots on MS 2,4- D, 1and BA, 1 static culture after 20 days.
E. Suspension culture of *P. anisum* calli with aerial parts containing MS BA 1liquid medium after 5 weeks of culture initiation.
F. Suspension culture of *P. anisum* calli with aerial parts and roots containing MS BA 1liquid medium after 1 month of culture initiation.

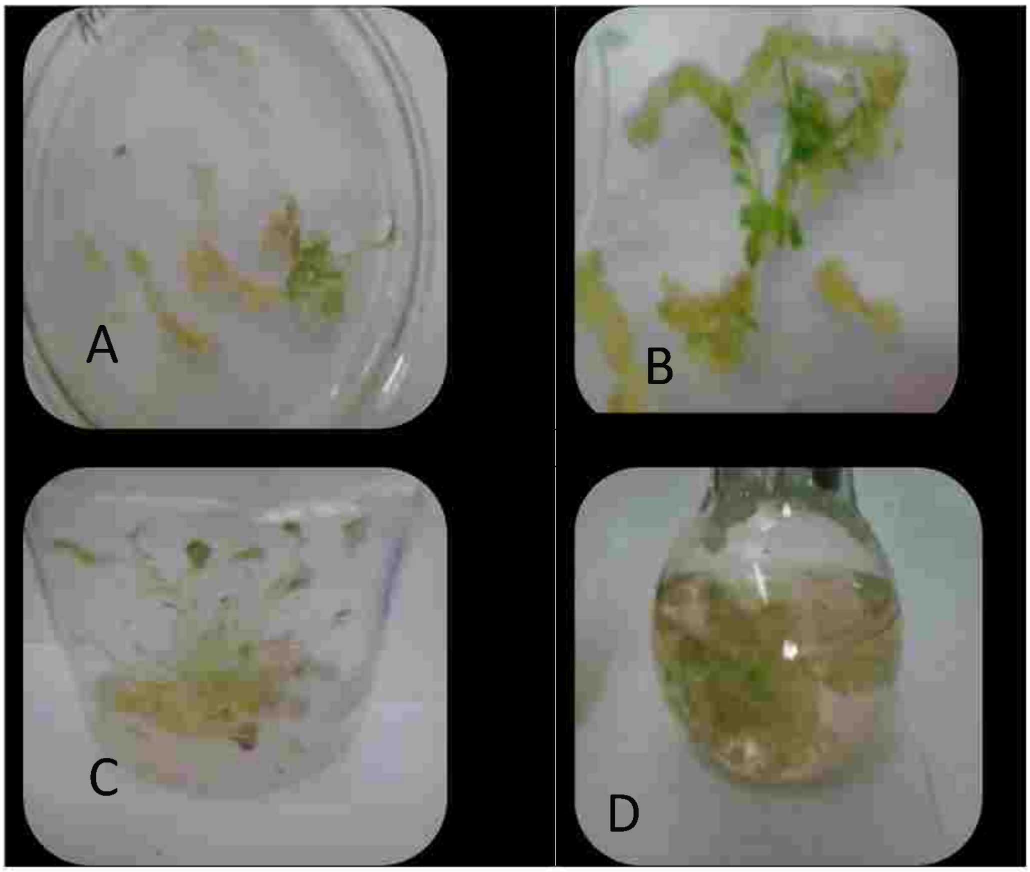


Figure7. Formation of *P. anisum* L. callus in a hormonal combination of 2, 4- D, 1 and KN, 1 static culture.

A. *P. anisum* callus with small shoots and leafy structure on MS 2,4- D, 1and KN, 1 static culture after 20 days.

B. *P. anisum* callus with increased shooting and leafy structure on MS 2,4- D, 1and KN, 1 static culture after 1 month.

C. *P. anisum* callus with branched aerial parts on MS 2,4- D, 1and KN, 1 static culture after 5 weeks.

D. Suspension culture of *P. anisum* calli with aerial parts containing MS BA,1 liquid medium after 6 weeks of culture initiation.

Figure 8. Micrograph of *O. basilicum* calli showing cytodifferentiated hairs (40X).

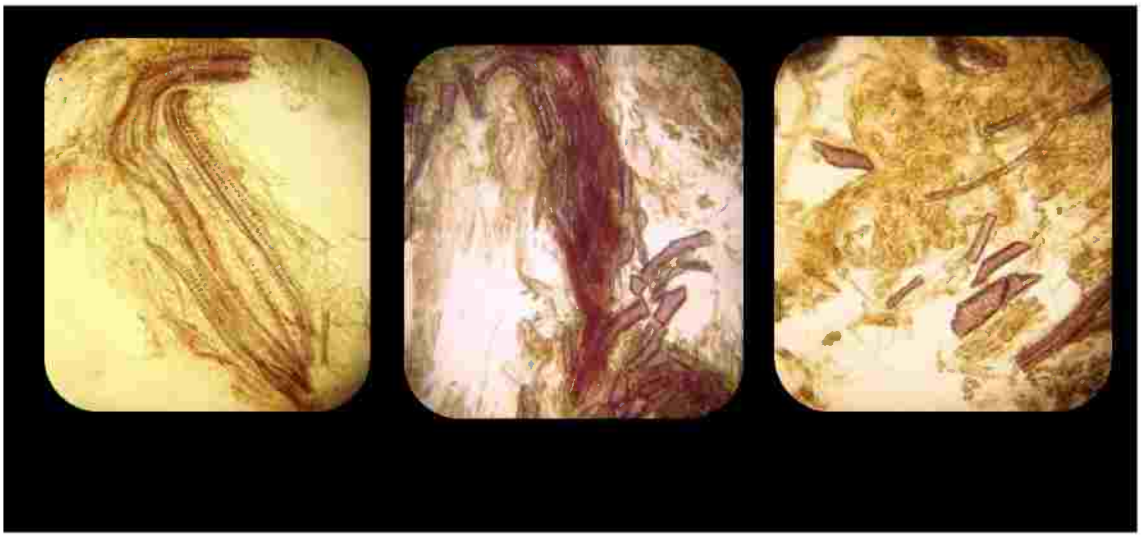


Figure 9. Micrograph of *P. anisum* L. calli showing cytodifferentiated vessels (Xylogenesis) (40X).

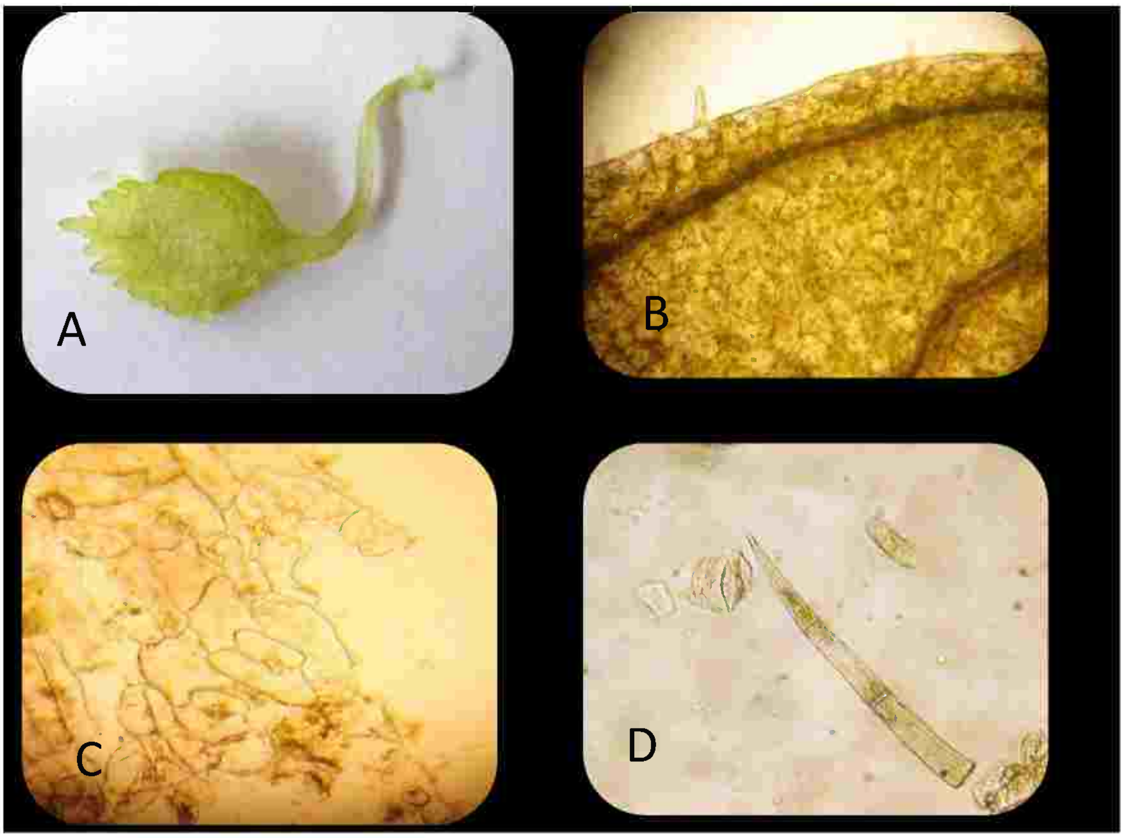


Figure 10. *P. anisum* L. calli showing cytodifferentiation and regenetated leafy structure (organogenesis).

- **A.** Leaf formed from calli of *P. anisum* with 1.5 cm length, 1.2 cm width, and a stalk length of 1.8 cm.
- **B.** Leaf formed from calli of *P. anisum* with observed venation, stomatal cells and hairs (40X).
- **C.** Stomatal cells in *P. anisum* leaf (paracytic stomata) (40X).
- **D.** Non – glandular hair formed in *P. anisum* (40X).

Results and discussion

Static and suspension cultures

i. *O. basilicum* L.

The leaves were used as explants for static culture. MS medium was used as the basal tissue culture nutrient supplemented with (KN, 1) and (2, 4-D, 1) or with mixture of (BA, 1) and (2, 4-D, 1) growth hormones.

A. (2,4- D, 1) and (BA, 1) combination

Yellowish white callus of a contact mass initiated on the periphery of cut surface exposed to the media after approximately 2 weeks. After one month all the leaf explants converted into a light brown callus. The formed calli gradually were converted into a dark brown colour, mostly due to oxidation of their phenolic content. The formed calli were transferred into a liquid media containing (BA, 1) as a growth regulator; formation of aerial parts was noticed sometimes after 10 days of suspension culture initiation [Figure 3].

B. (2,4- D, 1) and (KN, 1) combination

Yellowish white callus of a contact mass initiated on the periphery of cut surface exposed to the media after approximately 20 days. After approximately 45 days all the leaf explants converted into a yellowish brown callus that was slightly had a dark brown colour with time. The formed calli were transferred into a liquid media containing (BA, 1) as a growth regulator [Figure 4]. It was noticed that this combination of hormones has a slower and less pronounced effect on callus formation than the first combination. Also, its phenolic content may be lower than the first combination. Therefore, the best protocol for induction of a good quality callus was a combination of (2, 4-D, 1) and (BA, 1) in a static culture and only (BA, 1) in suspension culture.

ii. *P. anisum* L.

Seeds were firstly germinated on MS as a basal nutrient medium. The produced hypocotyls after one month were aseptically transferred to MS medium supplemented with (2, 4-D, 1) to induce callus formation and with (KN, 1) or (BA, 1) as a type of cytokinin to induce shoot system.

A. (2,4- D, 1) and (BA, 1) combination

Yellowish green callus of a friable mass initiated on the periphery of hypocotyls explants exposed to the media after approximately 2weeks, and then formation of aerial parts (leaves, shoots) was noticed after 20 days. At the 30th day the produced aerial parts increased with high degree of branching and cytodifferentiation. Sometimes roots appeared in this culture combination, this may be due to increased concentration of *in vitro* cytokinins in the medium which stimulated endogenous auxins of the plant to initiate roots. The produced calli with aerial parts and sometimes roots were transferred into a liquid media containing (BA, 1) as a growth regulator [Figure 6].

B. (2,4- D, 1) and (KN, 1) combination

Yellowish green callus of a friable mass initiated on the periphery of hypocotyls explants exposed to the media after approximately 20 days, then formation of aerial parts (leaves, shoots) was noticed after one month. After about 5 weeks of culture initiation the produced aerial parts increased with high degree of branching and cytodifferentiation. The produced calli with aerial parts were transferred into a liquid media containing (BA, 1) as a growth regulator [Figure7].

So, the first hormonal combination of (2,4- D, 1) and (BA, 1) in static culture and only BA, 1 in suspension culture offers more favorable conditions for faster callus initiation and organogenesis.

Microscopical examination of the produced calli

i. *O. basilicum* L.

Microscopical examination of *O. basilicum* calli revealed the formation of non-glandular, multicellular hairs contain 3-4 cells, slightly curved but sometimes appear as a whip- shaped hair having enlarged basal cell. Both forms of hairs covered with warty cuticle, and having a pointed end, and that was indicative for the occurrence of cytodifferentiation [Figure 8].

ii. *P. anisum* L.

Microscopical examination of *P. anisum* calli revealed the presence of lignified vessels (xylogenesis). The vessels are characterized by different lignification patterns like spiral, annular, perforated and sclariform [Figure 9].

The calli showed the development of leafy structure, with the following botanical description:

- **i.** Light green Broad and ovate lamina with 1.2 cm width and 1.5 cm length.
- **ii.** Dentate margin.
- **iii.** Acute apex.
- **iv.** Reticulate venation.
- **v.** Petiolate with a petiole length of 1.8 cm.
- **vi.** Has a symmetric base.

Microscopical examination of *P. anisum* formed leafy structure showed cytodifferntiation represented by formation of stomatal cells (paracytic stomata), and hairs which described as non-glandular, tricellular, straight hairs and covered with warty cuticle [Figure 10].