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Research Article

ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *NIGELLA SATIVA* L. FOR THYMOL PRODUCTION

HERA CHAUDHRY[1], NIDA FATIMA[2] AND IFFAT ZAREEN AHMAD[1*]

Department of Bioengineering, Integral University, Dasauli, Kursi Road, Lucknow 226026, Uttar Pradesh, India.
Email: iffat77@rediffmail.com, iffat77@gmail.com

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ABSTRACT

Objective: *Nigella sativa* Linn. is a small elegant annual herb and is regarded as one of the greatest form of healing medicine available. Thymol, one of its pharmaceutically sound bioactive compound has been reported to possess anti inflammatory, antioxidant, anticancerous and anti-neoplastic effects. This study was done to investigate callusing response from different parts of the *in vitro* grown seedlings and production followed by qualitative determination of thymol through suspension culture initiated by the produced callus.

Method: Callusing response of different parts (leaf, epicotyl, hypocotyl and root) of the *in vitro* grown seedling was determined on solid MS media with different growth hormones so as to generate inoculums for suspension culture. Suspension culture was initiated in liquid MS media supplemented with different hormones. Effect of these hormones on total terpenoid production was estimated followed by thymol estimation through thin layer chromatography.

Results: Epicotyl region inoculated on MS media supplemented with Kn (2 mg/L) + NAA (1 mg/L) gave fast growing creamy and friable callus in comparison to different parts of the seedlings on different combinations used. Suspension cultures with Kn (2 mg/L) + NAA (1 mg/L) showed the maximum growth rate and biomass accumulation followed by BAP (2 mg/L) + IAA (1 mg/L). Variations in hormone combination and concentration showed an increased percentage of polar and non-polar terpenoid (11.5% non-polar and 4% polar) over the standard suspension culture. Further, thin layer chromatography of these cultures revealed the presence of thymol with a characteristic pink spot of R_f value 0.76 in cultures with Kn (2 mg/L) + NAA (1 mg/L) and BAP (2 mg/L) + IAA (1 mg/L).

Conclusion: It could be concluded from the study that combination of Kn (2 mg/L) + NAA (1 mg/L) and BAP (2 mg/L) + IAA (1 mg/L) enhanced the *N. sativa* terpenoid production and also induced greater production of thymol in suspension culture.

Keywords: Callus, Suspension, Thymol, Growth regulators.

INTRODUCTION

Medicinal plants have been a major source of therapeutic agents since ancient times to cure human diseases. Despite the major advances in the modern medicines, the development of new drugs from natural products is still considered important. In this regard, one such plant is *N. sativa* Linn. which is a small elegant herb believed to be endogenous to the Mediterranean region but has been cultivated in other parts of the world including India and Pakistan [1]. It is regarded as one of the greatest forms of healing medicine available. Studies have revealed various therapeutic values of *N. sativa* such as anti-inflammatory, anti-analgesic, anti-stress, anticancer, antioxidant, antibacterial, antifungal, antiparasitic and antiasthmatic [2, 3, 4]. The vast therapeutic characteristics of *N. sativa* seeds are due to their unique phytochemical composition [5, 6]. Humans use these secondary metabolites as medicines, flavorings, and recreational drugs. Secondary metabolites are ''high value low volume'' products [7, 8]. The volatile oil of the seed (0.5–1.6%) is composed mainly of the monoterpenes *p*-cymene, γ-terpinene, α-pinene, β-pinene, α-thujene, carvacrol, thymol and thymoquinone [9, 4]. Volatile oil of the black seeds imparts them with the anti-histanimic, antioxidant, anti-infective and bronchodilating types of pharmaceutical activities. Thymol, is one of the active compound in *N. sativa* extract, plays important role in the inhibition of cancer cells, antibacterial activity against oral bacteria also, anti inflammatory activity, fungicidal activity, carcinogenicity and can attach with the mutagenic substance, because thymol is one of the antioxidant phenolic compound.

Therefore, *in vitro* produced cultures can be used an alternative for meeting out the demand of secondary metabolites within reasonable time and obtain them in large amount. Plant tissue culture refers to growing and multiplying of the cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Tissue culture derived material provides industrial source of different necessary metabolic compounds such as alkaloids, phenols, terpenoids, vitamins and other of compounds which are of medicinal value [10].

Generally used methods of plant tissue cultures for the production or enhancement of the plant products include callus culture and suspension culture mainly. It has been shown by the researchers that callus formation is governed by the source of explants, nutritional composition of the medium and environmental factors [11]. An important milestone in the callus culture is the role of auxin and cytokinin interaction. Suspension culture on the other hand is a sterile (closed) system normally initiated by aseptically placing friable callus fragments into a suitable sterile liquid medium [12, 13].

In view of this, the present investigation on tissue culture of *N. sativa* was established *in vitro* to elucidate the response of different explants towards callusing and effect of different growth regulators on callus production under defined nutritional, hormonal and cultural conditions. Further the calli was used to initiate the suspension cultures in order to study the effect of growth regulators on growth rate, percentage terpenoid production and qualitative determination of thymol.

MATERIALS AND METHODS

Plant Material

Seeds of *N. sativa* were grown in glass petri plates having two or three folds of damp blotting paper in distilled water at room temperature of about 25±2^{0}C under aseptic condition so as to get sufficient sterile inoculums. The complete germination took eleven days with green leaves, epicotyl, hypocotyl, and root. The seeds were incubated in dark till sprouting was initiated (3 days) after which the plates were transferred to culture room at a light intensity of 100 µmol m^{-2} $s^{-1}$and a 14/10 h (day/night) photoperiod till the complete plantlet with two leaves were obtained.

Callus Induction and Maintenance

Sterilized leaf, segments (0.5 cm^2) of epicotyl, hypocotyl and root were cultured on solidified MS medium supplemented separately with the combinations of Kn 2mg/L + NAA 1 mg/L, BAP 2 mg/L + IAA 1 mg/L, Kn 2mg/L + IAA 1mg/L and BAP 2 mg/L + NAA 1 mg/L and a control. Each combination was taken in a triplet. Cultures were kept under a photo period of 14-10 with 3000 lux of white fluorescent light at 25+2°C temperature in the culture room. Initiation of callus started after 7-10 days of culturing and the pattern of the growth of callus was observed. Data were recorded after 4 weeks on fresh weight of callus.

Establishment of cell suspension cultures and growth measurement

Cell suspension cultures were derived from friable epicotyl callus in Erlenmeyer flasks (250 ml) each containing 100ml of liquid MS medium supplemented with different combinations of cytokinins and auxins (Kn 2mg/L + NAA 1 mg/L, BAP 2 mg/L + IAA 1 mg/L, Kn 2mg/L + IAA 1mg/L and BAP 2 mg/L + NAA 1 mg/L and control). Each combination was taken in triplets. The cell suspensions were maintained at 110 rpm on a rotary shaker at 25±2C, 3000 lux and 16/8 h photoperiod. Cultures were maintained for a month and their growth was determined by loss of weight by dissimilation. [14].

Percentage estimation of terpenoids in suspension culture

Estimation of intracellular terpenoids of the suspension cultures was done following the method of Banhtrope [15]. 2-3 gm of the suspension cells were taken and exhaustively extracted with methanol and water mixture (4:1). Each extract was filtered with Whatman no.1 filter paper. Residue was discarded and filtrate was reduced to 1/10 at 40°C on water bath. Concentrated extract was then extracted with chloroform water mixture (1:1). Chloroform and aqueous layers were separated and evaporated till dry residue was obtained. Total non-polar and polar terpenoids were calculated by using following formulae,

$$\text{Total non-polar terpenoids} = \frac{\text{Weight of Chloroform residue}}{\text{Amount of sample taken}} \times 100$$

$$\text{Total polar terpenoids} = \frac{\text{Weight of Aqueous residue}}{\text{Amount of sample taken}} \times 100$$

Qualitative estimation of thymol in suspension cultures

Extracts used for loading silica gel TLC plates were prepared by redissolving separately chloroform and aqueous residues in 90% of methanol. 10-20µl of samples (both chloroform and aqueous residues) were separately loaded on TLC plates developed in a solvent containing toulene:chloroform:ethanol (40:10:10). Plates were sprayed with freshly prepared anisaldehyde-sulphuric acid reagent. Plates were then heated to 100°C for 10 min and R_f values of the spots were measured.

RESULTS

Response of the explants towards callusing

Explants inoculated in triplets in glass test tubes with 15-20 ml of solidified MS media were demonstrated for the callus production which showed that the epicotyl segments (0.5 cm long) gave the best and fast response (Table 1) with white to creamy friable callus which started in second week of the culture and covered the entire length of the explants within 28-30 days, followed by leaf disc segments (0.5 cm long) also showed good callusing response, gave compact, green callus but callusing here was delayed when compared to epicotyl whereas root and hypocotyls segment gave no response towards callusing (Figure 1).

Table 1: Response of different explants of *N. sativa* seedling towards callusing

S. No.	Days	Explants			
		Leaf	Epicotyl	Hypocotyl	Root
1	7	+	++	-	-
2	14	++	+++	-	-
3	21	+++	++++	-	-
4	28	++++	+++++	-	-

* '-' symbolizes no response towards callusing; *'+' symbolizes response towards callusing; *'++' symbolizes moderate response; *+++ symbolizes good response.

Epicotyl region **Leaf segment** **Hypocotyl region**

Root segment

Fig. 1: Callusing response of different explants of *N. sativa.*

Effect of growth regulators on callusing

Callusing was induced only when the combination of cytokinin and auxin were used. No sign of callus formation was seen in the presence of only cytokinin or auxin. The callus formation showed best response in the presence of high cytokinin and low auxin. Cytokinins like benzyl amino purine (BAP) and kinetin (KN) were tried. Auxins used for the induction of callus were naphthalene acetic acid (NAA) and indole acetic acid (IAA). Callus from different explants showed varied response when cultured under different growth regulator regimes. Percentage of callusing per explant was calculated through fresh weight and it was maximum in MS medium with Kn (2.0 mg/L) + NAA (1.0 mg/L) (Table 2) up to 82.6% in epicotyl which gave creamy, fragile and fast growing callus. Callusing response was also good in the case of leaf explants inoculated on media with BAP (2.0 mg/L) + IAA (1.0 mg/L) which was 69.6% (Table 3) but here callus produced was compact and growth was also not as fast as in the case of epicotyl. Callusing response in Kn (2 mg/L) + IAA (1 mg/L) and BAP (2 mg/L) + NAA (1 mg/L) was late and the callus formed was compact i.e. not a desirable material for initiating suspension culture whereas there was diminished callusing in the control tubes without any hormone. It took about 4-5 weeks for callusing over entire explants.

Table 2: Effect of growth hormones on nature and percentage of callus induction from the epicotyl segment of *N. sativa*

Growth regulator	Concentration (mg/l)	Nature of callus	Percentage of callus
MS media + Kn	1.0	No response	-
	2.0	No response	-
	3.0	No response	-
MS media+Kn +NAA	1.0+1.0	Creamy, compact	73.4±0.08
	2.0+1.0	Creamy, friable	82.6±0.17
	3.0+1.0	Creamy, friable	75.3±0.33
MS media+BAP+IAA	1.0+1.0	Creamy, friable	60.4±0.10
	2.0+1.0	Creamy, compact	72.6±0.03
	3.0+1.0	Creamy, compact	55.3±0.23
MS media+Kn+IAA	1.0+1.0	Creamy, friable	28.3±0.54
	2.0+1.0	White, friable	25.1±0.08
	3.0+1.0	White, friable	32.4±0.12
MS media+BAP+NAA	1.0+1.0	Creamy, friable	22.7±0.23
	2.0+1.0	White, friable	48.0±0.26
	3.0+1.0	White, friable	50.2±0.66
MS media+BAP	1.0	No response	-
	2.0	No response	-
	3.0	No response	-

Table 3: Effect of growth hormones on nature and percentage of callus induction from the leaf of *N. sativa*

Growth regulator	Concentration (mg/l)	Nature of callus	Percentage of callus
MS media + Kn	1.0	No response	-
	2.0	No response	-
	3.0	No response	-
MS media+Kn +NAA	1.0+1.0	Green, compact	63.4±0.08
	2.0+1.0	Green, compact	67.6±0.01
	3.0+1.0	Green, compact	56.3±0.39
MS media+BAP+IAA	1.0+1.0	Green, friable	50.4±0.20
	2.0+1.0	Green, compact	69.6±0.03
	3.0+1.0	Creamy, compact	42.3±0.63
MS media+Kn+IAA	1.0+1.0	Green, compact	13.9±0.98
	2.0+1.0	Green, compact	18.1±0.48
	3.0+1.0	Creamy, friable	27.4±0.12
MS media+BAP+NAA	1.0+1.0	No response	-
	2.0+1.0	Creamy, friable	36.0±0.76
	3.0+1.0	Creamy, friable	41.2±0.67
MS media+BAP	1.0	No response	-
	2.0	No response	-
	3.0	No response	-

Effect of growth regulators on the growth of suspension cultures

Batch suspension cultures (each combination in triplet including control) were initiated with 2-3 gm of epicotyl friable callus as an inoculum at temperature 25±2°C having photoperiod of 16-8 at 110 rpm in liquid MS medium supplemented with the same combinations of the growth regulators as those used in callus culture (Kn 2mg/L + NAA 1 mg/L, BAP 2 mg/L + IAA 1 mg/L, Kn 2mg/L + IAA 1mg/L and BAP 2 mg/L + NAA 1 mg/L) and control gave transparent, homogeneous and nonchlorophyllous cultures which were used to demonstrate the effect of plant growth regulators (PGR's) on growth of biomass. Growth pattern of each culture was determined by loss of weight dissimilation method, it showed that all cultures including control gave sigmoid curves same as the pattern followed by the suspension cultures with varying but all five growth phases i.e. lag, exponential, linear, stationary and progressive decline. The growth rates of cells were initially slow in the lag phase but as the cultures proceeded, the growth increased significantly and accumulated a great amount of fresh weight (5 fold) over a period of 15-20 days then the growth of cells became stable and started declining from 25^{th}-26^{th} day (Figure 2). Maximum growth was achieved in suspension culture supplemented with Kn (2mg/L) + NAA (1 mg/L) followed by Kn (2mg/L) + IAA (1mg/L) and then by BAP (2 mg/L) + IAA (1 mg/L), BAP (2 mg/L) + NAA (1 mg/L), with Kn (2mg/L) + NAA (1 mg/L) having the longest stationary phase whereas in the case of BAP (2 mg/L) + NAA (1 mg/L) stationary phase was shortest followed by rapid decline phase.

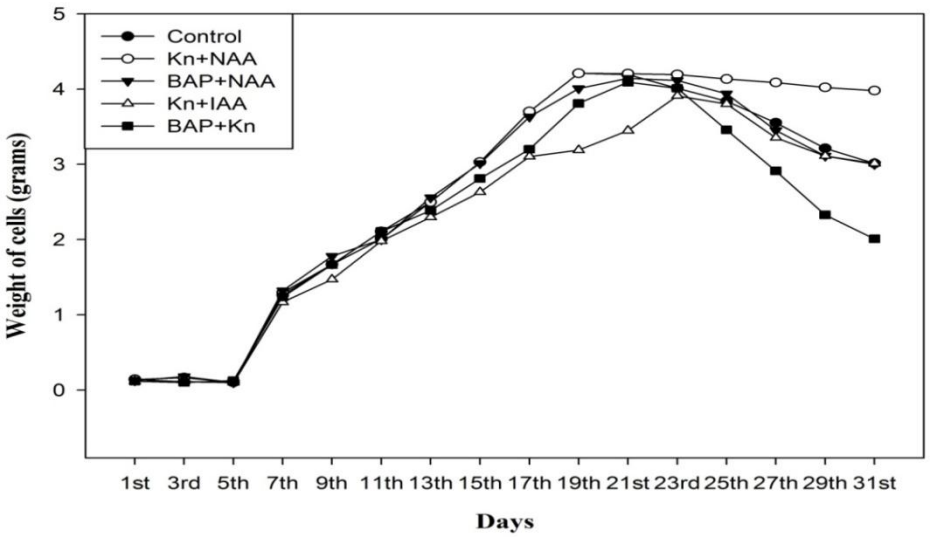

Fig. 2: Effect of different growth regulators on the biomass growth of suspension cultures

Effect of growth regulators on terpenoid production

In view of the positive effect of plant growth regulators on biomass and secondary metabolite accumulation, cells harvested after one month from different suspension cultures including control were subjected to estimate the effect of growth hormones on the intra cellular terpenoid content as the major secondary metabolites of *N. sativa* belong to this class. Percentage of both polar and non-polar terpenoids were estimated and the results showed that the addition of growth hormones increased the percentage of both polar and non-polar terpenoids lowest up to 2-4 folds as compared to the control i.e. like in the case of Kn(2mg/l) + NAA(1mg/l) combination total percentage of non-polar and polar terpenoids was 11.5% and 4% respectively followed by BAP(2mg/l) + IAA(1mg/l) combination where percentage for non-polar got reduced to 9.7% and percentage of polar terpenoids were 9% (Figure 3). In the case of other two combinations i.e. Kn(2 mg/l) + IAA(1 mg/l) and BAP(2 mg/l) + NAA(1 mg/l) the percentage was found to be 7%, 10% and 8.05%, 6.77% for non-polar and polar terpenoids respectively. All of these were much higher than the total non-polar and polar terpenoid percentage calculated in control where it was 3% and 1.5% respectively. This clearly showed a makeable increase in terpenoid content which was in regard to the addition of PGR's.

Effect of growth regulators on thymol production

The results (Table 4) illustrate that the addition of different PGRs to suspension cultures lead to the production or even enhanced production of thymol which was qualitatively estimated by thin layer chromatography. Results showed that TLC of chloroform residue samples prepared from the harvested cells of suspension culture grown with the combinations of Kn(2 mg/l) + NAA(1 mg/l) and BAP(2 mg/l) + IAA(1 mg/l) gave a characteristic pink spot of 0.76 R_f value similar to that of standard used i.e. thymol. On the other hand Kn(2 mg/l) + IAA(1 mg/l) gave a diminished pink spot due to low abundance of thymol. Whereas this spot was not seen in the case of control and also in the combination of BAP (2 mg/l) + NAA (1 mg/l). Further a blue spot of R_f value 0.35 was seen in all cases including control but the intensity of spot was higher in Kn(2 mg/l) + IAA(1 mg/l) and BAP(2 mg/l) + NAA(1 mg/l) and the intensity of the spot was low in the case of Kn(2 mg/l) + NAA(1 mg/l), BAP(2 mg/l) + IAA(1 mg/l) and control indicating presence of other terpenoid. TLC results of aqueous residue samples showed that only KN (2 mg/l) + NAA (1 mg/l) gave a diminished pink spot similar to standard with R_f value 0.76 whereas the spot was totally absent in other sample (Table 5). Presence of blue spot of low degree was seen in combinations of Kn (2 mg/L) + IAA (1 mg/L) and in BAP (2 mg/L) + NAA (1 mg/L).

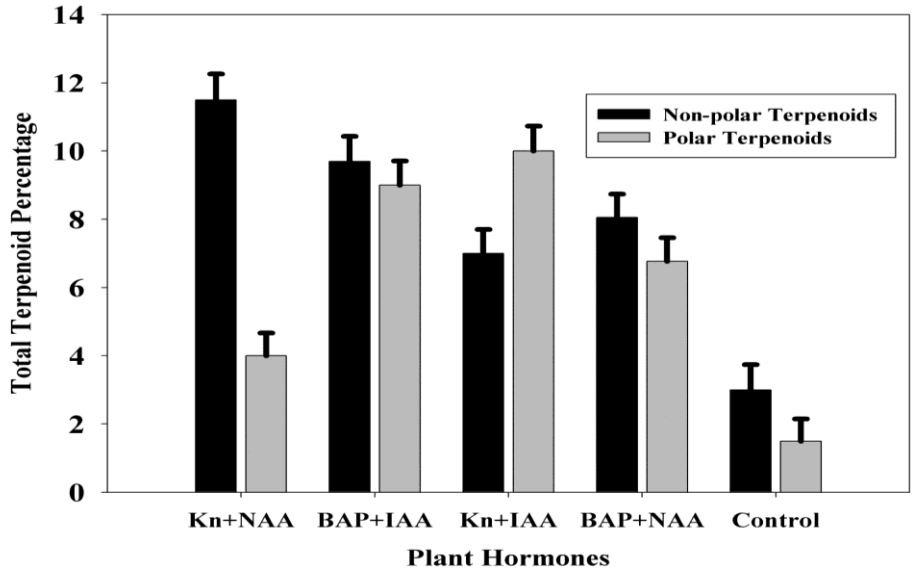

Fig 3: Effect of growth hormones on total percentage of terpenoid (polar and non-polar terpenoids) production in different suspension cultures.

Table 4: TLC of chloroform residue samples prepared from different suspension cultures showing the effect of growth hormones on thymol production.

S. No.	Color	R_f values	Std Thymol	Hormone combinations				Control
				Kn+NAA	BAP+IAA	Kn+IAA	BAP+NAA	
1	Pink	0.76	+	+++	++	+	-	-
2	Blue	0.35	-	+	+	++	++	+

* '-' symbolizes absence of the metabolite; *'+' symbolizes presence of the metabolite; *'++' symbolizes moderate presence; *+++ symbolizes good presence, *Std: standard.

Table 5: TLC of aqueous residue samples prepared from different suspension cultures showing the effect of growth hormones on thymol production.

S. No.	Color	R_f values	Std Thymol	Hormone combinations				Control
				Kn+NAA	BAP+IAA	Kn+IAA	BAP+NAA	
1	**Pink**	**0.76**	**+**	**+**	**-**	**-**	**-**	**-**
2	**Blue**	**0.35**	**-**	**-**	**-**	**+**	**+**	**-**

* '-' symbolizes absence of the metabolite; *'+' symbolizes presence of the metabolite; *Std: standard.

DISCUSSION

Juvenile explants for the initiation of *in vitro* cultures are primarily favored because of their high morphogenetic potential and low level of contamination [16]. On the contrary, establishing *in vitro* cultures of plant using mature tissues as explants could not be always accomplished, mainly due to the high contamination rates, reduction or absence of morphogenetic ability and poor rooting of regenerated shoots therefore in the present study we used the different parts of the *in vitro* grown seedling of *N. sativa* so as to maintain the sterility level also. Keeping in view, the response of the different explants towards induction of callus it was seen that callusing was best in epicotyl followed by leaf explant and no response was seen in root and hypocotyl segment. Thus callus induction was found to be tissue-dependent and similar to the results reported in callus induction of *Orthosiphon stamineus*, in which it was described that callus could be induced successfully from leaf, petiole, stem but not root [17]. This difference is dependent upon the response of plant tissues to various growth promoting substances. Besides the type, age and genotype of the explants, successful callus induction depends upon the various factors such as compositions of the nutrient medium, hormone balance [18]. However, the recent investigations have examined the process involved in the supply of minerals *in vitro* and suggested a complex web of interactions between the explants and culture medium [19, 20].

Callusing response and the amount of callus produced from the explants depend on many factors including balancing of exogenous and endogenous auxin-cytokinin levels. Furthermore, MS medium supplemented with 2, 4-D, NAA, BAP & Kn were utilized by previous researcher for *in vitro* cultures of several species [21]. Results of the present study clearly showed that only Kn and BAP gave no response and as it has already being stated in the literature that high exogenous auxins are essential for development of the callus from any explant [22] therefore combination of Kn (2 mg/L) + NAA (1mg/L) and BAP(2 mg/L) + IAA (1mg/L) gave best callus percentage calculated from the fresh weight in the case of epicotyls callus which was 82.6% and 72.6%, respectively. This showed that high concentration of both the auxins i.e. NAA and BAP along with the cytokinins were required to produce the callus. Cytokinin or auxin alone do not support callus formation whereas in the case of control diminished growth was reported with 17.24% of callus. Morphology of the callus also varied with different plant growth regulators used in the medium as stated in the result, which differed from creamy friable to compact in case of the epicotyl callus whereas leaf callus were green or creamy compact.

It has been reported in past literature that actively growing and friable callus material were successfully used for *V. vinifera, B.vulgaris, L.esculentum, L. erythrorhizon, Pueraria lobata* (Willd) cell cultures to produce anthocyanins, betalains, carotenoids, naphtoquinones, isoflavones respectively [23, 24, 25, 26, 27] keeping in view these cited literatures the friable and actively growing epicotyl calli were taken as the inoculating material for the suspension culture and not the compact calluses of the both leaf and epicotyl. The results of the present study have shown that cell growth requires a certain initial density of cells and lower inoculum size is inhibitory to growth of suspension cultures therefore inoculum size of 2-3 gm was taken to initiate the suspension culture. Similar growth inhibition has also been reported for transformed cell suspension cultures of *Coleus forskohlii* [28]. The inoculum size had a positive effect on biomass and metabolite production up to an optimum concentration [29]. In this study, the optimal inoculums size for biomass accumulation and the production of terpenoids was 2-3% (FW, w/v), which is comparable to those obtained by Chiou [30] for production of polyunsaturated fatty acids in cell suspension cultures of *Marchantia polymorpha*. Growth hormones like cytokinins and auxins play an essential role in coordination of growth processes in plant cell suspensions. They are considered as the key regulators in sustaining growth of suspension cells. Nevertheless, as it was stated by Teale [31] that individual cell cultures differ in their sensitivity to these substances. Hence, it is necessary to determine their appropriate concentrations. Further, Ramachandro and Ravishankar [32] cited that in cell suspensions, since the culture is homogenous, the action of exogenous substances, like growth regulators, may have a stronger effect. In this study, we have tested synergistic effects of four plant hormones i.e. Kn(2 mg/L) + NAA(1 mg/L), BAP(2 mg/L) + IAA (1 mg/L), Kn (2 mg/L) + IAA (1 mg/L) and BAP (2 mg/L) + NAA (1 mg/L) on the cellular growth of the suspension culture cells which was determined by loss of weight dissimilation method results showed that Kn along with the auxins either NAA or IAA gave maximum biomass accumulation as compared to BAP and control. All cultures along with the control followed the typical sigmoid growth curve.

The ancient Egyptians, Greeks and Romans were already aware of the therapeutic properties of *N. sativa*, the essential oil and seeds of which are still used in folk medicine, as a flavouring agent and as a spice in various kinds of meals. By investigating the previous studies which have shown monoterpenes, including thymol, *p*-cymene, α-thujene, γ-terpinene, carvacrol, α-pinene and β-pinene, to be the main components of the essential oil from black cumin [4, 9]. However, these studies also state that there are still unidentified components [4, 9]. Thymol (2-isopropyl-5-methylphenol) ($C_{10}H_{14}O$) being a natural monoterpene phenol which has been isolated from the essential oils of various medicinal plants [33]. Previous research results have claimed it also as a main constituent of *N. sativa* seed [34]. In several research studies different bioactivities and pharmacological properties of *N. sativa* seeds were found to be due to thymol which include anti-oxidant [35] anti-microbial [36, 37] and anti-inflammatory [38] activities. Studies show that thymol has

also been reported to possess anti-mutagenic, anti-tumor [39] and fungicide [40] effects. Therefore as because of its bioactivities this study aimed at the production and enhanced production of the thymol through plant tissue culture. Suspensions of the epicotyl callus of *N. sativa* were cultured under the varying concentrations and combinations of the auxins and cytokinins so as to determine their effect on the production and enhancement of the secondary of metabolites of *N. sativa* mainly thymol. As secondary product accumulation might be dependent on the development of specialized cells and not purely on the growth and proliferation. Hence extended stationary phase of *N. sativa* cells in suspension culture showed the highest production of the both polar and non-polar terpenoids as shown in the results. It is known that at the growing phase, the cellular enzymes are repressed and supplemented carbon sources and growth regulators are not utilized for the biosynthesis of the compounds [41]. Past works prove that maintaining cells under stationary phase is very important towards the elicitation of secondary compounds [42]. As the growth curve of the Kn (2 mg/L) + NAA (1 mg/L) entered the stationary phase earliest of all and was the longest one therefore supporting the fact of highest amount of polar and non-polar terpenoids production by these cells as they have to starve through a longer period of time as compared to other cultures under other combinations of plant hormones.

Further the effectiveness of the whole study was tested through qualitative analysis of all the suspension cultures via thin layer chromatography for the presence of thymol. Results of the chloroform residues marked the presence of thymol in Kn (2 mg/L) + NAA (1 mg/L) and in BAP (2 mg/L) + IAA (1 mg/L) by a pink color spot as that in the standard when compared with the standard thymol used. Synergistic effect of BAP (2 mg/L) + IAA (1 mg/L) gave a less prominent pink spot suggesting a less abundance of thymol in it. R_f values calculated corresponded with the values given in the literature cited by Hepsibah [43] which came to be 0.76 in the case of the two hormonal synergisms and 0.76 for standard thymol. This spot was absent in the case of control and other two combinations used suggesting that they do not influence the production of thymol. Further the TLC analysis of the aqueous residues gave low results with only Kn (2 mg/L) + NAA (1 mg/L) combination showed diminished presence this is mainly because as thymol get better extracted/dissolved in non polar solvents much better than in water. Presence of blue spot of low degree was seen in combinations of Kn (2 mg/L) + IAA (1 mg/L) and in BAP (2 mg/L) + NAA (1 mg/L).

CONCLUSION

The present world is surrounded by many dreadful diseases and cure of these diseases needs a prolonged treatment which itself sooner or later leads to mall functioning of some organs. Therefore, the present study was undertaken which showed the enhancement of important metabolites of *N. sativa* by varying the concentration of growth hormones in callus and suspension culture. These may be used as an alternative to sort out the above mentioned problems as the metabolites of *N. sativa* have shown tremendous medicinal and pharmaceutical properties. Hence, it could be concluded by these findings that we can enhance these pharmaceutically important compounds easily which can be used as natural drugs in future.

REFERRENCES

1. Chakraverty HL. Plant Wealth of Iraqi Dictionary of Economic Plants. Vol I. Baghdad: 1976. p. 387-588.
2. Islam MH, Ahmad IZ and Salman MT. *In vivo* evaluation of anti-inflammatory and analgesic activities of *Nigella sativa* seeds during germination. Int J Pharm Pharm Sci 2013; 5 (4): 451-454.
3. Roshan S, Abdullah Khan, Tazneem B and Sadath Ali. To study the effect of *Nigella sativa* on various biochemical parameters on Stress induced in albino rats. Int J Pharm Pharm Sci 2010; 2: 185-189.
4. Burits M and Bucar F. Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 2000; 14: 323-328.
5. Padhye S, Banerjee S, Ahmad A, Mohammad R and Sarkar FH. From here to eternity-the secret of Pharaohs: Therapeutic potential of black cumin seeds and beyond. Cancer Ther 2008; 6: 495–510.
6. Ali BH and Blunden G. Pharmacological and toxicological properties of *Nigella sativa*. Phytother Res 2003; 17: 299–305.
7. Katare DP, Aeri V, Bora M. Secondary metabolites and metabolic engineering. J Cell Tissue Res 2009; 9(3): 2027–2036.
8. Babula P, Adam V, Havel L and Kizek R. Noteworthy secondary metabolites naphthoquinones their occurrence, pharmacological properties and analysis. Curr Pharm Anal 2009; 5: 47–68.
9. Benkaci-Ali F, Baaliouamer A, Meklati BY and Chemat F. Chemical composition of seed essential oils from Algerian *Nigella sativa* extracted by microwave and hydrodistillation. Flavour Frag J 2007; 22: 148–153.
10. Shengwei Z and Jingsam S**.** Rapid plant regeneration from cotton *Gossypium hirsutum* L. Chin Sci Bulletin 2000; 45(19): 1772-1773.
11. Arora A and Chawla HS. Organogenic plant regeneration via callus induction in Chickpea (*Cicer arietinum* L.) - Role of genotypes, growth regulators and explants. Ind J Biotech 2005; 4: 251-256.
12. Dixon RA. Isolation and maintenance of callus and cell suspension cultures. In: Dixon RA, editor. Plant Cell Culture; a Practical Approach. IRL Press; 1985. p. 1–20.
13. Gamborg OL and Phillips GC. Media preparation and handling. In: Gamborg OL, and Phillips GC, editors. Plant Cell, Tissue and Organ Culture; Fundamental Methods. Springer Lab Manual, Springer-Verlag; 1995. p. 1–90.
14. Schripsema J and Verpoorte R. Novel techniques for growth characterization and phytochemical analysis of plant cell suspension cultures. J Nat Prod 1995; 58: 1305–1314.
15. Dey PM and Harborne JB. Terpenoids. In: Banthorpe DV and Charlwood BV editors. Methods in plant biochemistry. London, UK: Academic Press. 1991; 7: p. 43-44.
16. Yang ZS, Chen GD, Li YX and Chen J. Characterization of callus formation in leaf of Euphorbia helioscopia. Afr J Plant Sci 2009; 3: 122-126.
17. Lee WL and Chan LK. Establishment of *Orthosiphon stamineus* cell suspension culture for cell growth. Plant Cell Tiss Org 2004; 78: 101-106.
18. Huang HC and Murashige T. Plant tissue culture media, major constituents, their preparation and some applications. Tissue Culture Association Manual 1976; 3: 539-549.
19. Williams RR. Mineral nutrition *in vitro* A mechanistic approach. Aust J Bot 1993; 41: 237-251.
20. Williams RR. The chemical micro-environment. In: Aitken-Christie J, Kozai T and Smith MAL editors. Automation and environmental Control in Plants Tissue Culture. Dordrecht: Kluwer Academic Publishers; 1995. p. 405-439.
21. Catapan L, Silva B, Moreno FN and Viana AM. Micropropagation, callus and root culture of *Phyllanthus urinaria* (Euphorbiaceae). Plant Cell, Tissue Organ Culture 2002; 70: 301-309.
22. Evans DA, Sharp WR and Flick CE. Growth and behavior of cell culture: Embyrogenesis, organogenesis. In: Thrope TA editor. Plant Tissue Culture Methods and Applications in agriculture. New York: Academic Press; 1981. p. 45-113.
23. Pepin MF, Archambault J, Chavarie C and Cormier F. Growth kinetics of *Vitis vinifera* cell suspension cultures: Shake flask cultures. Biotechnol Bioeng 1995; 47: 131-138.
24. Klebnikov, Dubuis AB, Kut OM and Prenosil JE. Growth and production of *Beta vulgaris* cell culture in fluidized bed reactors. Bioprocess Eng 1995; 14: 51-56.
25. Fosket D and Radin D. Induction of carotegenesis in cultured cells of *Lycopersicon esculentum*. Plant Sci Lett 1983; 30: 165-175.
26. Sim SJ and Chang HN. Increased shikonin production by hairy roots of *Lithospermum erythrorhizon* in two-phase bubble column reactor. Biotechnol Lett 1993; 15: 145-150.
27. Chen G and Li L. Nutrient consumption and production of isoflavones in bioreactor cultures of *Pueraria lobata* (Willd). J Environ Biol 2007; 28: 321-326.
28. Mukherjee S, Ghosh B, and Jha S. Establishment of forskolin yielding transformed cell suspension cultures of *Coleus forskohlii* as controlled by different factors. J Biotechnol 2000; 76: 73-81.

29. Gorret N, bin Rosli SK, Oppenheim SF, Willis LB, Lessard PA, Rha C, and Sinskey AJ. Bio- reactor culture of oil palm (*Elaeis guineensis*) and effects of nitrogen source, inoculum size, and conditio- ned medium on biomass production. J Biotechnol 2004; 108: 253-263.
30. Chiou SY, Su WW and Su YC. Optimizing production of polyunsaturated fatty acids in *Marchan- tia polymorpha* cell suspension culture. J Biotechnol 2001; 85: 247-257.
31. Teale WD, Paponov IA and Palme K. Auxin in action: Signaling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 2006; 7: 847-859.
32. Ramachandro RS and Ravishankar GA. Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 2002; 20: 101-153.
33. Morrison RT and Boyd RN. Organic Chemistry 6th Edn. New Delhi, India: Prentice Hall of India Private Limited 2003.
34. Al-Saleh AI, Billedo G and Al-Doush II. Levels of Selenium, DL-α-tocopherol, DL-γ- tocopherol, all-trans-retinol, thymoquinone and thymol in different brands of *Nigella sativa* seeds. Journal of Food Composition and Analysis 2006; 19(2-3): 167-175.
35. Kruk I, Michalska T, Lichszteld K, Kladna A and Aboul-Enein HY. The effect of thymol and its derivatives on reaction generating reactive oxygen species. Chemosphere 2000; 41(7): 1059–1064.
36. Bourgou S, Pichette A, Marzouk B and Legault J. Bioactivities of black cumin essential oil and its main terpenes from Tunisia. South African J Bot 2010; 76(2): 210-216.
37. Salman MT, Khan RA and Shukla I. Seed oil against multi-drug resistant bacteria from clinical isolates. Nat Prod Radi 2008; 7(1): 10-14.
38. Marsik P, Kokoska L, Landa P, Nepovim A, Soudek P and Vanek T. *In vitro* inhibitory effects of thymol and quinones of *Nigella sativa* seeds on cyclooxygenase-1- and -2-catalyzed prostaglandin E2 biosyntheses. Planta Medica 2005; 71(8): 739-42.
39. Andersen A. Final report on the safety assessment of sodium p-chloro-m-cresol, p- chloro-m-cresol, chlorothymol, mixed cresols, m-cresol, o-cresol, p-cresol, isopropyl cresols, thymol, o-cymen-5-ol, and carvacrol. Int J Toxicol 2006; 25(1): 29-127.
40. Ahmad A, Khan A, Yousuf S, Khan LA and Manzoor N. Proton translocating ATPase mediated fungicidal activity of eugenol and thymol. Fitoterapia 2010; 81(8): 1157-1162.
41. Raval NK, Hellwig S, Prakash G, Ramos PA, Srivastava A and Buchs J. Necessity of a two stage process for production of azadirachtin related limonoids in suspension cultures of *Azadirachtin indica.* J Biosci Bioeng 2003; 96: 16-22.
42. Fliescher A, Titel C and Ehwald R. The boron requirement and cell wall properties of growing and stationary suspension cultured *Chenopodium album* L. cells. Plant Physiol 1998; 117: 1401-1410.
43. Hepsibah PTA, Prasad NBR and Sanjeev Kumar P. Standardisation of Ayurvedic Oils. Ancient Science of Life 1998; 17(4): 280 – 283.