Plant Tissue Cult. & Biotech. **18**(2): 181-185, 2008 (December)

PTC&B

# Thymol Production from Callus Culture of *Nigella sativa* L.

**Nabeel K. Al-Ani**

*Biotechnology Department, College of Science, University of Al Nahrain, Iraq*

*Key words:* Thymol produiction, Callus culture, *Nigella sativa*

## Abstract

Roots, hypocotyls and leaves of *Nigella sativa* L. were collected from the seedlings raised on sterilized filter paper and cultured on MS supplemented with different concentrations of 2,4-D (0.0, 1.0, 2.0, 3.0, 4.0 mg/l) and Kn (0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0). The best callus production was obtained from leaf explants with 1 mg/l 2,4-D and 1.5 mg/l Kn. The higher thymol concentrations were extracted after 75 days for the above callus; which was detected by HPLC using retention time. This is the first report in Iraq about extracting thymol from callus of *Nigella sativa*.

## Introduction

*Nigella sativa* L. belongs to the Ranunculaceae family comprising many annual herbs. In Arab countries its seeds are known under different name such as Al-Habbah Al-Sawda, Kamoun Aswad, Scuniz and Kodria. The famous saying (Hadith) of our Prophet Mohammed (PBUH); that "Habbat Al-soda" is remedy for all diseases except death prompted us to undertake this piece of investigation. Among reported biological activities (Ansari et al. 1988, some are: (a) it is anti-inflammatory, (b) anti-tumorous (Worthen et al. 1998, Detommasi et al. 2000), (c) it relaxes the central nervous system, (d) it acts against stroke and (e) it reduces sugar and cholesterol level in the blood (Bingham et al. 1978). Thymoquinone and dithymoquinone, the active principles of *N. sativa,* were found to possess cytotoxic effects against parental and multi-drug resistant human tumour cell lines which were over tenfold more resistant to doxorubicin and etoposide (Poter et al. 1975). Thymol, thymoquinone, carvacol, anthole and terpinol are the active compounds of black seed oil, which act as antioxidant compounds in *N. sativa* (Burits and Bucar 2000, Kruk et al. 2000). This experiment was carried out to find an alternative method for thymol production, and in view to biotechnologically manipulate the *in vitro* culture.

Micropropagation of medicinal plants by tissue culture provides a quicker and better technique of mass production of raw source material over conventional method. 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 (Shengwei and Jingsam 2000).

## Materials and Methods

The mature seeds of *Nigella sativa* L. were taken from Agriculture Research and Food Technology, Ministry of Science and Technology, Baghdad, Iraq. Seeds were sterilized by soaking for five min in 50% normal bleach which contains sodium hypochloride (NaOCl) (3%), and rinsed three times in sterile distilled water. Seeds were then germinated on Petri dishes lined with filter paper soaked with several ml of sterile distilled water in light room. Leaves, hypocotyls, and roots were separated and cultured in MS containing different concentrations of Kn (1, 1.5, 2, 2.5, 3 and 5 mg/l) and 2,4-D (1, 2, 3 and 4 mg/l), 30% sucrose, 1% casein hydrolysate and solidified with agar. The pH of the media was adjusted to 5.7 prior autoclaving. The explants were grown at 25 ± 1$^{o}$C with a photoperoid of 16 hrs light. The subcultures were carried every three weeks in the same media and growth conditions.

The callus was established from three weeks old plants germinated *in vitro*. The callus was maintained in MS with 1 mg/l of 2,4-D and 1.5 mg/l of Kn and subcultured every three weeks. The production of thymol was estimated at 75th day.

Thymol from 75 days old leaf callus was extracted and isolated according to (Harvath 1980) Thymol was identified and quantified by comparing it with the pure compound as a standard. Thymol was quantified using HPLC technique as described by the separation of thymol occurred on a reversed phase de base colum (MDB) 250 × 4.6 mm internal diameter (ID). The mobile phase that is used buffer phosphate: methanol (30 : 70), the pH was adjusted to 3.5. The detection occurred at UV light at 254 nm wave length with flow rate at 1 ml/min.

## Results and Discussion

The seeds were sterilized with in 3% NaOCl for 5 min., then washed three times with sterilized distilled water, then sown in sterile Petri dishes on filter papers. After germination the roots, hypocotyls, and leaves were removed and cultured on MS supplemented with different concentrations of 2,4-D as an auxin and Kn. The 2,4-D concentrations were 0, 1, 2, 3 and 4 mg/l, while Kn concentrations were 0, 1, 1.5, 2, 2.5, 3 and 5 mg/l. The results for leaf explants in Table 1 indicate that 2, 4-D above 3 mg/l had no affect on callus formation. The highest mean of callus weight of 1400 mg was obtained in MS supplemented with 1 mg/l 2,4-D and 1.5 mg/l Kn (Table 1). These results were in agreement reported by Voet and Voet (1991).

The results (Table 2) refer red to the callus formation from hypocotyls explants. The results indicate no significant differences between different 2,4-D concentrations unless Kn was introduced.

**Table 1. Fresh weight (mg) of 75 days old leaf callus of *N. sativa* with different auxin- and cytokinin concentrations.**

| Kn (mg/l) | 2,4-D (mg/l) | | | | | Mean ± SE |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | |
| 0 | 0.0 ± 0.0 a | 1000 ± 104 b | 1000 ± 99.9 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 400 ± 244.9 a |
| 1 | 1000 ± 144.3 a | 500 ± 95.2 b | 810 ± 212.2 ab | 0.0 ± 0.0 c | 0.0 ± 0.0 c | 462 ± 204.8 a |
| 1.5 | 250 ± 50.3 a | 1400 ± 359.2 b | 740 ± 151.4 ab | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 478 ± 267.1 a |
| 2 | 0. 0 ± 0.0 a | 900 ± 45.8 b | 140 ± 26.4 c | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 208 ± 175.1 a |
| 2.5 | 500 ± 202 ab | 710 ± 200.1 a | 90 ± 28.8 b | 0.0 ± 0.0 b | 0.0 ± 0.0 b | 260 ± 145.6 a |
| 3 | 0.0 ± 0.0 a | 600 ± 5.77 b | 90 ± 40.9c | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 138 ± 116.8 a |
| 5 | 0.0 ± 0.0 a | 400 ± 120.9 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 80 ± 80 a |
| Mean ± SE | 250 ± 144.3 a | 787.1 ± 129.7 b | 410 ± 159 ab | 0.0 ± 0.0 a | 0.0 ± 0.0 a | |

Differences a, b and c ($p < 0.05$ ) for comparison between rows and columns.

**Table 2. Fresh weight (mg) of 75 days old callus from hypocotyls segments.**

| Kn (mg/l) | 2,4-D (mg/l) | | | | | Mean ± SE |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | |
| 0 | 1500 ±173.2 a | 1000 ± 125.8 ab | 800 ± 246.6 b | 0.0 ± 0.0 c | 0.0 ± 0.0 c | 660 ± 292.5 a |
| 1 | 1000 ± 388.3 a | 250 ± 28.8 b | 560 ± 20.8 ab | 0.0 ± 0.0 b | 0.0 ± 0.0 b | 362 ± 189.8 ab |
| 1.5 | 0.0 ± 0.0 a | 220 ± 30 ab | 430 ± 160.9 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 130 ± 86.2 b |
| 2 | 0.0 ± 0.0 a | 200 ± 36 b | 280 ± 90.7 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 96 ± 60.1 b |
| 2.5 | 0.0 ± 0.0 a | 160 ± 45.8 ab | 190 ± 80.2 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 70 ± 43.1 b |
| 3 | 0.0 ± 0.0 a | 150 ± 28.8 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 30 ± 30.1 b |
| 5 | 0.0 ± 0.0 a | 150 ± 28.8 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 30 ± 30 b |
| Mean ± SE | 357.1± 236.9 a | 304.2 ± 116.8 a | 322.8 ± 111.6 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | |

Differences a, b and c ($p < 0.05$ ) for comparison between rows and columns.

The reasons behind these responses may be due to the use of explants when using high concentrations of cytokinins especially Kn that make cells grow without divisions and this is partially related to water absorption because of the formation of the reduced saccharides by the cells following lipid transformation during cytokinensis (Kliss et al. 1973)

Table 3 shows that different concentrations of Kn gave no significant differences in callus mean weight while significant differences were found when different concentrations of 2,4-D were used.

The concentration 1 mg/l of 2,4-D gives highest mean weight of callus mass i.e., about 741.4 mg when compared with other concentrations of 2,4-D (3 and 4 mg/l) which were not responsive to callus formation. This may be due to higher concentrations of auxin proving toxic to callus cells either deceasing or inhibiting its growth (Raven et al. 1986 )

**Table 3. Fresh weight (mg) of 75 days old callus from root segments.**

| Kn (mg/l) | 2,4-D (mg/l) | | | | | Mean ± SE |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | |
| 0 | 710 ± 149.7 a | 0.0 ± 0.0 b | 0.0 ± 0.0 b | 0.0±0.0 b | 0.0 ± 0.0 b | 142 ± 141.9 a |
| 1 | 1000 ± 144.3 a | 300 ± 28.8 b | 250 ± 76.3 b | 0.0 ± 0.0 c | 0.0 ± 0.0 c | 310 ± 183.3 a |
| 1.5 | 933.3 ± 33.1 a | 1000 ± 57.7 b | 220 ± 30 b | 0.0 ± 0.0 c | 0.0 ± 0.0 c | 430.7 ± 222 a |
| 2 | 0.0 ± 0.0 a | 900 ± 86.6 b | 190 ± 37.8 c | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 218 ± 174.4 a |
| 2.5 | 0.0 ± 0.0 a | 700 ± 208.1 b | 100 ± 25.6 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 160 ± 136.4 a |
| 3 | 0.0 ± 0.0 a | 300 ± 140 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 60 ± 60 a |
| 5 | 0.0 ± 0.0 a | 100 ± 80 b | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 20 ± 19.9 a |
| Mean ± SE | 377.6 ± 236.9 ab | 471.4 ± 149.1 b | 108.5 ± 42.1 ab | 0.0 ± 0.0 a | 0.0 ± 0.0 a | |

Differences a, b and c ($p < 0.05$ ) for comparison between rows and columns.

The HPLC was used to detect the presence of thymol in the leaf callus explants. The retention time in these samples was 5.358 and 5.517 for the samples and standard thymol, respectively.

## References

**Ansari AA, Hassan S, Kenne L, Rahman A.** and **Wheeler T** (1988). Structural studies on saponin isolated from *Nigella sativa*. Phytochemistry **27**(12): 3977-3979.

**Bingham R, Harris DH** and **Laga T** (1978) Yucca plant saponin in the treatment of hypertension and hypercholesterolemia. J. Appl. Nutr. **30**: 127.

**Burits M.** and **Bucar F** (2000). Antioxidant activity of *Nigella sativa* essential oil. Phytother. Res. **14**(5): 323-328.

**Detommasi N, Autor G, Beilino A, Pinto A, Pizza G** and **Sorrention R** (2000) Antimproliferative triterpene saponins from *Trevesia palma*. J. Nat. Prod. Mar. **63**(3): 308-14.

**Harvath C** (1980) High performance liquid chromatography advances and perspectives. Academic Press. New York. London, Toronto, Sydney, San Francisco.

**Kliss FM, Groot C** and **Verwer R** (1973) Effect of carbon sources, gibberellins and ethylene on wall - bound invertase activity in callus of *Convolvulus arvensis*. Physiol. Plant. **30**: 334-336.

**Kruk I, Michalska T, Lichszteld K, Klanda A** and **Aboul-Enein HY** (2000) The effect of thymol and its derivatives on reactions generating reactive oxygen species. Chemosphere **41**: 1059-1064.

**Poter JD, Topping DL** and **Oakenfull DG** (1975) Soya products, saponins and plasma cholesterol. Lancet **1**: 223.

**Raven PH, Evert RF** and **Eichorn SE** (1986) Biology of Plants 4th ed, Worth Publishers.

**Shengwei Z** and **Jingsam S** (2000) Rapid plant regeneration from cotton (*Gossypium hirsutum* L.). Chin. Sci. Bulletin **45**(19):1772-1773.

**Voet D** and **Voet J** (1991) Biochemistry. New York, Chichester, Brisbane, Toronto, Singapore.

**Worthen DR, Ghosheh OA** and **Crooks PA** (1998) The *in vitro* antitumor activity of some crude and purified components of black seed. *Nigella sativa* L. Anticancer Res. **18**(3A): 1527-31.