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Research Article

Elicitation of Thymol in *Thymus Vulgaris*, A Medicinally Important Plant

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Abstract

Thymus vulgaris is a Mediterranean plant that is known for its aroma present maximally in the leaves, stem and purple flowers. The aromatic is due to the essential oil that is rich in monoterpenes and its derivatives. The major active ingredient that is present in the essential oil is thymol.. This study involves the screening of substances that increase the concentration of thymol in the plant using biotic and abiotic substances. The abiotic elicitors screened were 1mM acetic acid, manganese chloride, silver nitrate, salicylic acid and methyl jasmonate; while the biotic elicitors used were 1 g/l *Aspergillus niger* cell free extract and yeast extract. The effect of the elicitor on thymol concentration in the plant was evaluated for 24, 48 and 72 h. The elicitation of thymol was detected and quantified using HPTLC and densitometric quantification software after extraction of thymol in Dichloromethane (DCM). The concentration of thymol in the unexposed plants measures 35 mg/g dry weight of the plant and that of the best abiotic elicitor, Silver nitrate is 90mg/g. Thus the highest amount (three-fold increase compared to the control) of thymol accumulation was observed in the shoots after 48 hours of exposure to silver nitrate.

Keywords Thymol, silver nitrate, elicitation, HPTLC fingerprint, *Thymus vulgaris*.

Introduction

Thymus vulgaris, belongs to lamiaceae family and is a Mediterranean shrub that has a pleasant aroma[1]. The aroma is due to essential oils present in fresh leaves and purple flowers. They are used as aroma additives in food, pharmaceuticals and cosmetics[2]. The major active ingredient that is present in the essential oil is thymol[3]. Thymol (2-isopropyl-5-methylphenol) is a monoterpene phenol predominantly present in the essential oil of *Thymus sps*, *Origanum sps*, *Monarda sps*, *Trachyspermum sps*, etc. It is a white crystalline substance with an aromatic odour and antiseptic properties. It has antifungal potential against *Aspergillus parasiticus*, antioxidant effect and antimicrobial activity[2]. The total yield of thyme essential oil is much less and hence the amount of thymol is also less. Hence to increase the essential oil content and thus the thymol, Elicitation is one of the tools that can be used.

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It is well known that different stresses, locations, climates, microenvironment and physical and chemical stimuli (often called elicitors) quantitatively and qualitatively alter the content of bioactive secondary metabolites[4]. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival persistence and competitiveness. Elicitors are molecules that stimulate the plant defense mechanism. They are compounds of biological or non-biological origin, which upon contact with plant cells; trigger defense related compounds through over-expression of relevant enzymes[5,6]. These defense-related responses are activated through a signal transduction pathway that includes the recognition of elicitors by receptors located in the plasma-membrane, activation of ion fluxes across the membrane, induction of down-stream functions such as oxidative bursts (free radical formation) and formation of secondary messengers[7]. Due to such high specificities of actions, failure of one elicitor does not necessarily mean that the metabolic pathway cannot be triggered indicating that a large number of elicitors have to be screened for accomplishing effective elicitation process. Hence in the current investigation seven different elicitors have been screened, of which two are biotic and five are abiotic in nature. A number of analytical techniques have been developed to determine the different components present in plant extracts or essential oil obtained from plants. Chromatographic fingerprinting of phyto-constituents can be used for the assessment of quality, stability and comparison of the standardized fingerprint pattern to new or modified fingerprints obtained after certain treatment to the plants[8]. They can also be used for quantification of the obtained fingerprint using densitometric analysis.

MATERIALS AND METHODS

2.0 gm of shoots of *T. vulgaris* L obtained from the wild were placed in a 250 mL tissue culture bottle containing 50 mL of Hoagland's medium[9] and incubated at 25 °C on a gyratory shaker. The shoots were harvested from the media after 24, 48 and 72 hrs. These shoots served as control. To evaluate the effect of various elicitors, the shoots were exposed to each of elicitors by adding each elicitor at fixed concentration into the medium and incubating at 25 °C on a gyratory shaker. The shoots were harvested after 24, 48 or 72 hrs of exposure.

The biotic elicitors used for elicitation were Yeast extract (YE) and mycelial extract (also called dry cell powder) of *Aspergillus niger* (AnE) at a concentration of 1g/L. Yeast

extract powder was purchased from Himedia. The culture of *A. niger* was obtained from NCL, Pune. They were maintained by routine sub culture on Sabouraud's agar slants. The dry cell powder of *A. niger* was prepared for elicitation at a concentration of 1 g/L in basal medium (Hoagland's Medium)[10]. The complete elicitation medium was autoclaved at 121 °C at 1.05 Kg/cm^2 for 20 min before inoculation of the shoots of *T. vulgaris* L.

The abiotic elicitors were filter sterilized through a 0.2 µM bacteria-proof filter (Millipore) and added into sterile basal medium (Hoagland's Medium) at their respective concentrations. Different abiotic elicitors used in the basal media were 0.5 mM Acetic acid (HOAc), 0.5 mM Salicylic acid (SA), 0.5 mM Manganese chloride ($MnCl_2$), 0.5 mM Silver nitrate ($AgNO_3$) and 0.5 mM Methyl jasmonate (MeJa)

The harvested shoots were dried between sheets of filter paper at room temperature for 48 hrs. 0.1 gm dried plant material from each of the 24 samples were taken and crushed and subjected to solvent extraction using dicloromethane (DCM). The extract was resuspend in 500 µL of toluene and used for further analysis[11].

The standard thymol was procured from Loba Chemie and prepared at a concentration of 10 µg/mL (w/v) in toluene. 5 µL was used to load on aluminium baked thin layer chromatography (TLC) plate pre-coated with silica gel 60F254 (Merck). 12 samples of 10 µL were applied at a speed of 150 nL/sec on the plate as bands of 8 mm width using CAMAG Linomat V sample applicator. The mobile phase constituted of toluene-ethyl acetate in the ratio of 93:7 (v/v). After development the plate was dried and visualized under visible light, ultraviolet (UV) light at 254 nm and 366 nm before derivatization. The plates were derivatized by dipping them in vanillin-sulphuric acid (VS) reagent[11]. The fingerprint was evaluated in visible mode as well as UV at wavelength of 366 nm, using CAMAG Visualizer. Thymol appears as apink coloured spot after derivatisation. The plates were scanned at 530 nm using the WinCATS and VedioSCAN software to determine the area occupied by thymol zones.

RESULTS AND DISCUSSION

The shoot extracts obtained after extraction with DCM were green in colour. This is due to the simultaneous extraction of chlorophyll and accessory pigments present in the dried plant material along with all the non-polar substances. The TLC plates were visualized after development and before derivatization under visible light, UV light at 254 nm and UV light at 366 nm. The standard thymol did not show any zone, which indicates that thymol cannot be visualized under visible light or using ultra violet light. Hence derivatization was essential. After derivatization the plates were viewed under visible light as well as UV at 366 nm Figure 1. Thymol separated in the 'standard' lane as a single zone at Rf value of 0.43. Both the plates showed the presence of pink spots in all the lanes at Rf 0.43 indicating the presence of thymol in the control as well as the treated shoot extract.

The area under the peak obtained from the analysis of the plates represents the amount of thymol present in the zones after the plates were scanned using the CAMAG visualiser and density determined by the WinCATS software. The concentration of thymol obtained in terms of µg/gm of plant extracted for each treatment has been compared in *figure 2*. The control (untreated) shoots of *T. vulgaris* L shows the presence of 43.38 µg/gm, 28.5 µg/gm and 33.69 µg/gm of thymol after 24, 48 and 72 hrs of harvest. The maximum concentration (94.94 µg/gm) of thymol has been observed in shoots treated with 0.5 mM silver nitrate after 48 hours of elicitation, followed by 0.5 mM acetate (62.07 µg/gm).

From the current investigation, it can be concluded that 0.5 mM silver nitrate was successful in eliciting thymol at the highest concentration within 48 hrs of exposure. At 24 hrs and 72 hrs exposure, accumulation of thymol is low when silver nitrate is used to elicit. Silver nitrate has been proven to be a good abiotic elicitor for accumulation of Spiroketal enol ether diacetylenes in Feverfew hairy root cultures[12], lettucenin A in lettuce[13] and sakuranetin in paddy leaves[14]. The elicitor that successfully showed elicitation of thymol next in potency to silver nitrate is acetic acid at a concentration of 0.5 mM. Poulev *et al.*[4] have also shown elicitation of certain phytochemical compounds in different plant species using acetic acid as one of the abiotic elicitors. Visible browning of the media as well as the shoots was observed when acetate was used as the elicitor. This is indicative of enhanced phenolic oxidation which is sometimes also associated with cell death[6]. Hence silver nitrate is more effective as an abiotic elicitor because the plant shoots have not shown any visible damage or browning.

Among the biotic elicitors tested, the highest level of thymol was obtained when yeast extract was used as elicitor. When the shoots were exposed to 1 g/L yeast extract for 48 hrs, there was 2.7 times more thymol accumulation than the control shoots, compared to 1 g/L *Aspergillus niger* extract that recorded only 1.65 times more thymol accumulation. Ajungla *et al.*[15], report the superiority of yeast extract in elicitation of phytochemicals hyoscyamine elicitation in *Datura metel* L compared to *A. niger*. Yeast extract has been used for its ability to act as a biotic elicitor for elicitation of silymarin in *Silybum marianum*[16], Sanguinarine in *Eschscholtzia californica*[17] and sesquiterpenes in *Nicotiana tabacum*[18].

Salicylic acid and methyl jasmonate are known as signaling molecules during biotic and abiotic stress in plants. The percentage elicitation of thymol in shoots of *T. vulgaris* L was much less compared to the other elicitors used (silver nitrate, acetate, yeast extract and *A. niger* extract). They showed 1.5 and 1.7 fold increase

respectively in thymol accumulation compared to the control after 48 hours of exposure.

Manganese chloride at a concentration of 0.5 mM did not exhibit noticeable increase in thymol accumulation compared to control. The possible reason for no elicitation could be that the concentration of manganese chloride used was too less to induce any stress to the plants, as manganese is one of the nutrients required by plants for normal growth. Abiotic elicitation is hypothesized to involve a number of factors like the action by secondary messenger - Ca^{2+}, factors affecting cell membrane integrity, inhibition or activation of intracellular pathways and changes in osmotic pressure acting as stress agents[19].

A general mechanism for biotic elicitation in plants may be summarized on the basis of elicitor-receptor interaction. When a plant or plant cell culture is challenged by the elicitor an array of biochemical activities occur, which includes binding of the elicitor to a plasma membrane receptor or altered ion fluxes across the plant cell membrane. The ion flux is mediated by ions like Cl^- and K^+ efflux and Ca^{2+} influx. In plants, Ca^{2+} transients have been found to act as second messengers in a variety of responses to environmental signals, including pathogens. For instance, in parsley cells, an elicitor-responsive calcium channel has been identified and characterized and a transient influx of calcium has been found to occur within minutes after fungal elicitor addition.

An ubiquitous feature of plant response to pathogens or elicitors is the activation of phenylpropanoid metabolism in which phenylalanine ammonia-lyase (PAL) catalyses the first committed step of core pathway of general phenylpropanoid metabolism. Branch pathways lead to the synthesis of compounds that have diverse functions in plants, especially in defense such as cell wall repair, antimicrobial activity and as signaling compounds[6].

In case of *T. vulgaris* L, thymol (an antimicrobial agent) has been elicited in shoots when exposed to abiotic as well as biotic factors. Thus silver nitrate, acetate, yeast extract and *A. niger* extract, salicylic acid and methyl jasmonate may have been able to trigger the phenylpropanoid pathway thereby activating the cascade of events leading to increased biosynthesis and accumulation of thymol. The maximum exposure time, that recorded highest accumulation of thymol is 48 hrs for all the elicitors tested. The elicitors may be sprayed on the whole plant from where they are directly absorbed by the roots or the plant green parts like leaves. There are also reports of elicitation obtained when the cut leaves from the plant are exposed to elicitors in a liquid medium for 24-72 hours[20]. Applying elicitors to soil grown plants has serious limitations with poor uptake of chemical elicitors by the relatively impermeable hydrophobic surfaces of plant shoots[4]. Hence to overcome this limitation elicitors are generally applied to hydroponic system where the plants are grown in liquid medium. The roots are exposed to the medium, they do not have pigments and hence are easy to collect, dry, grind and extract the bioactive compound [4, 21].

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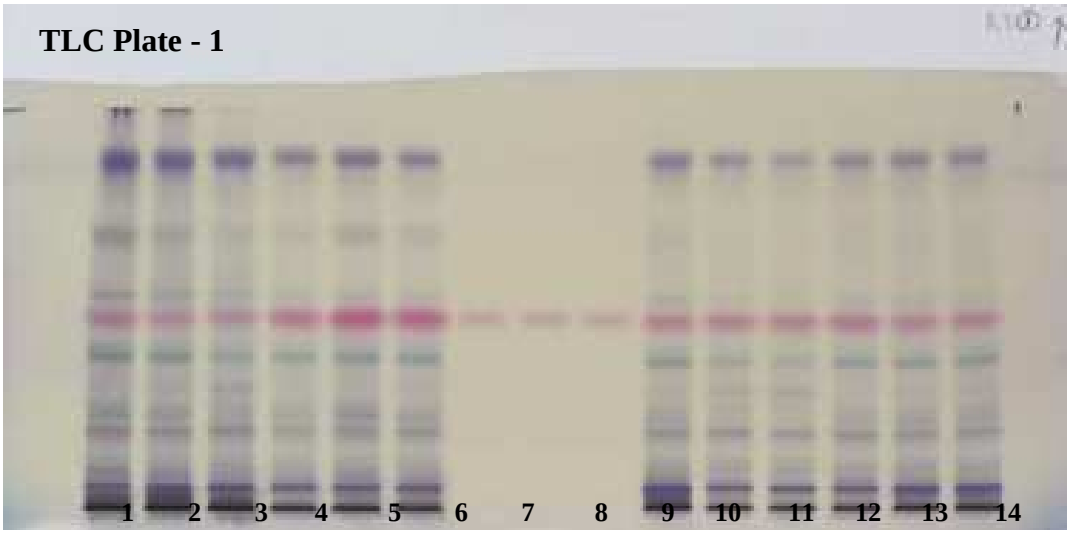
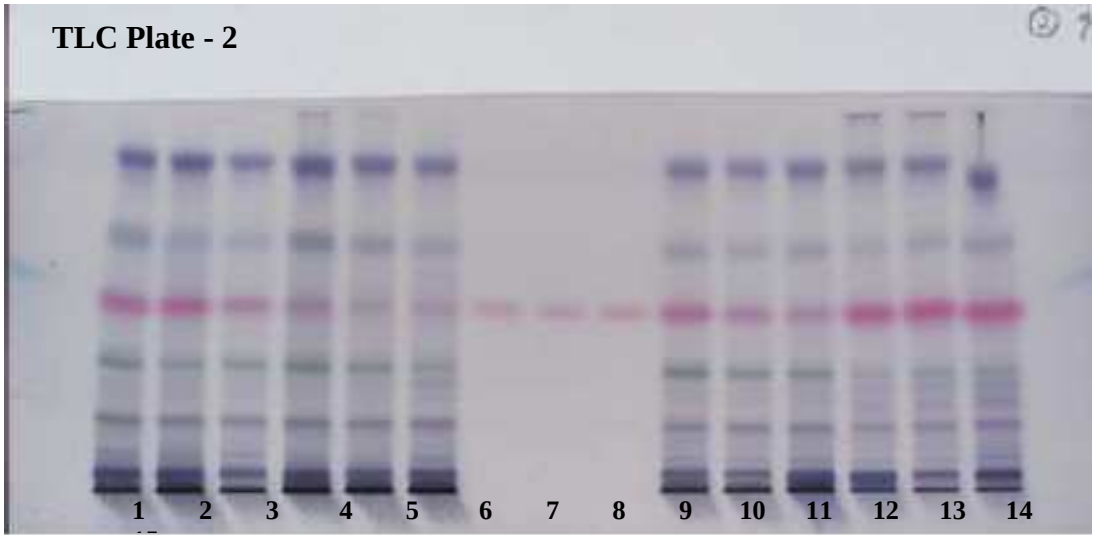
Figure 1
Observation of TLC plates under visible light after derivatization using VS reagent

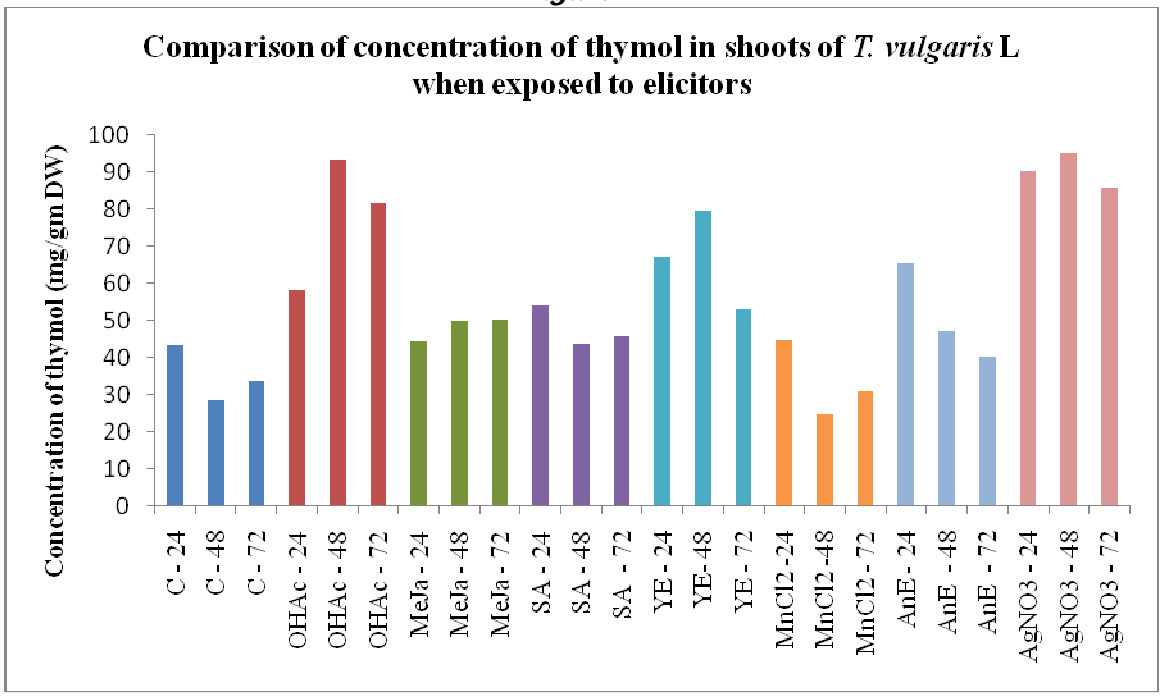


1 = C (24 hrs)	**4 = OHAc (24 hrs)**	**7 = Std Thymol**	**10 = MeJa (24 hrs)**	**13 = SA (24 hrs)**
2 = C (48 hrs)	**5 = OHAc (48 hrs)**	**8 = Std Thymol**	**11 = MeJa (48 hrs)**	**14 = SA (48 hrs)**
3 = C (72 hrs)	**6 = OHAc (72 hrs)**	**9 = Std Thymol**	**12 = MeJa (72 hrs)**	**15 = SA (72 hrs)**



1 = YE (24 hrs)	**4 = $MnCl_2$ (24 hrs)**	**7 = Std Thymol**	**10 = *An*E (24 hrs)**	**13 = $AgNO_3$ (24 h)**
2 = YE (48 hrs)	**5 = $MnCl_2$ (48 hrs)**	**8 = Std Thymol**	**11 = *An*E (48 hrs)**	**14 = $AgNO_3$ (48 h)**
3 = YE (72 hrs)	**6 = $MnCl_2$ (72 hrs)**	**9 = Std Thymol**	**12 = *An*E (72 hrs)**	**15 = $AgNO_3$ (72 h)**

Figure 2



Abbreviation:
C = Control
OHAc = acetic acid
MeJa = methyl jasmonate
SA = salicylic acid
YE = Yeast extract
$MnCl_2$ = Manganese chloride
AnE = Aspergillus extract
$AgNO_3$ = Silver nitrate
DW = Dry weight