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Investigation of limonene synthase quality articulation in various organs just as the impact of manganese on articulation of this quality in *Cuminum cyminum* L.

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Abstract

Semi-quantiative real time polymerase chain reaction (SQ-RT-PCR) was applied to study Limonene synthase (LS) gene expression pattern and the response to different concentrations of manganese in different organs of cumin (*Cuminum cyminum* L.). The results revealed that the gene was expressed in very small (< 2 mm) and small (3-4 mm) flowers and also in shoots, while it was not expressed in roots, leaves, medium (4-5 mm) and larger flowers. The highest level of gene expression was observed in shoot and very small flowers. Partial sequencing of LS gene in cumin revealed 68-88% identical to that of some other plants. To determine the effect of manganese on LS gene expression, the plants were exposed to different concentrations of manganese (0, 40, 80 and 160 ppm) as spray by applying two methods: T_1- Alternatively spraying at late vegetative stage besides blooming; T_2- Spraying only at blooming. LS gene expression was increased considerably under 80 ppm concentration of Mn at blooming and reduced remarkably under 40 ppm in both methods and at 160 ppm in T_1 method in comparison with controls. Anatomical studies indicated that essential oil ducts were located not only in the fruits tissue but also in the shoot surprisingly. Overall, results of this research reveal limonene synthase gene expression in cumin for the first time. In addition, use of 80 ppm of Mn at blooming can be considered as optimum concentration to increase LS gene expression. Existence of oil ducts on the shoot of this plant is a remarkable finding for further studies.

Keywords: *Cuminum cyminum* L., Limonene synthase gene, manganese.

INTRODUCTION

Medicinal and aromatic herbs have been receiving increased interest all over the world due to their efficacy as alternative medicine for curing human ailments without major known side effects. To date, very little is known about the genetics of plant secondary metabolism, as the genes of most pathways have not been identified and little is understood of their regulation and function. Cumin (*Cuminum cyminum* L.) is valued for its aroma, medicinal and therapeutic properties. The seeds contain 3–4% volatile oil and about 15% fixed oil (Spices Board Stastistics, 2006). In traditional medicine, cumin has various uses: it is used to treat hoarseness, jaundice, dyspepsia and diarrhoea. It is effective on gastrointestinal (Amin, 2000), reproductive (Weiss, 2002) and nervous (Janahmadi et al., 2006) systems. Cumin also has hypoglycaemic (Aslam et al., 2003), hypolipidaemic (Aruna et al., 2005) and chemoprotective (Gagandeep et al., 2003) properties. Terpenoids compose the largest and most diverse family of natural products. Of the more than 30,000 individual terpenoids now identified

Abbreviations: SQ-RT-PCR, Semi-quantiative real time polymerase chain reaction; **DNA,** deoxyribonucleic acid; **RNA,** ribonucleic acid; **MREs,** metal-responsive elements; **DMAPP**, dimethyl allyl pyrophosphate; **GPP**, geranyl pyrophosphate; **IPP,** isopentenyl pyrophosphate; **LS,** limonene synthase; **MEP,** methyl erythritol phosphate; **PBAF**, planting in peat moss with before and after flowering treatments; **PAF,** planting in peat moss with after flowering treatment; **T,** treatment.

(Buckingham, 1998), at least half are synthesized by plants. *C. cyminum* has limonene (Kan et al., 2007) which is a terpenoid. The synthesis of limonene, in providing the first committed intermediate of the pathway, represents a possible rate-limiting step of monoterpene production (Croteau and Gershenzon, 1994; Gershenzon and Croteau, 1990). It is assumed that monoterpenes (such as limonene) are primarily synthesized in the plastids via the methyl erythritol phosphate (MEP) pathway-derived isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) (Mahmoud and Croteau, 2002). Limonene serves as the common olefinic precursor of the essential oil terpenes (Kjonas and Croteau, 1983) through a series of secondary, largely redox, transformations (Croteau and Gershenzon, 1994). Limonene synthase enzyme catalyzes the stereo-specific cyclization of GPP to form the monocyclic monoterpene limonene (Munoz-Bertomeu et al., 2008). Limonene synthase (LS) gene has a key role in the beginning of biosynthesis pathway of terpenoids derivative compounds.

Manganese is an essential micronutrient throughout all stages of plant development (Marschner, 1995). This essential trace element plays an important role in several physiological processes as almost every compartment of the cell carries at least one enzyme whose activity is dependent on Mn^{2+}. This metal acts as cofactor for oxidases, dehydrogenases, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) polymerases, kinases, decarboxylases and sugar transferases (Crowley et al., 2000; Culotta et al., 2005; Keen et al., 2000). Many investigators have confirmed the role of elements on growth and yield of many aromatic and medicinal plants such as *Ruta graveolens* L. (El-Khateeb et al., 1994), *Mentha viridis* L. (El-Ghadban, 1994), Rosemary (Kassem, 2002) and *Trachyspermum ammi* L. (Swaefy, 2002). Manganese has been shown to be the most effective single micronutrient enhancing oil production (Nandi and Chatterjee, 1991). Manganese application as foliar spray (50 ppm) has been reported to increase the yield of essential oil and its main constituents in cumin (El-Sawi and Mohamed, 2002). Manganese-dependent gene expression is not fully understood in eukaryotic organisms. Existence of metal- or manganese-responsive sequences on promoter sequence of genes is possible (Gutierrez et al., 2008). Several heavy metal-inducible genes have been reported in plants (Berna and Bernier, 1999; Lescure et al., 1991; Hagen et al., 1988), but surprisingly, little is known about the transcriptional regulation of gene expression in response to heavy metals. Whilst there are some reports of the manganese effects on percent and components of oil in different plants, a few researches have been carried out on expression of precursor genes in this regard. The present investigation is the first attempt to study the limonene synthase gene expression in different organs as well as the effect of manganese on expression of this gene in *C. cyminum*. Also this paper studies existence of oil ducts in cumin shoot and flower.

MATERIALS AND METHODS

Plant materials and experimental design

Seeds of cumin (Cuminum cyminum L.) native to Neishabour city (Iran) were placed on water flow for 12-15 hours. Then, they were surface-sterilized by their immersion in 2% (v/v) NaOCl for 3 min and sulphur 80% DF fungicide for 2 min, followed by three times of rinsing in sterile water after any step. The seeds were next transferred to plastic pots containing (Klasmann-Deilmann, potgrond H) peat moss under equal greenhouse conditions (period of day/night, 16/8, at 26±3°C) and irrigated three times per week. Plants were affected by three methods: T0, without any treatment; T1, sprayed with different concentrations of manganese as $MnSO_4.4H_2O$ (0, 40, 80 and 160 ppm) both at late vegetative stage and blooming; T2, identical to T1 except for one spray only at blooming. Treatments were as follow: PBAF0, PBAF40, PBAF80 and PBAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese respectively, at late stages of vegetative phase and blooming; PAF0, PAF40, PAF80 and PAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese, respectively, only at blooming phase. For limonene synthase gene expression analysis in different organs, samples of the root, stem, leaf and flower at different developmental stages (F_1, <2 mm; F_2, 3-4 mm; F_3, 4-5 mm and F_4, >5 mm in size) were taken from T_0 method. These samples were immediately frozen in liquid nitrogen and stored frozen at -80°C until use. For LS gene expression analysis under manganese treatment, only F_2 flowers (3-4 mm) of plants in T_1 and T_2 methods were studied.

Determination of Mn content in *C. cyminum*

The aerial parts of plants in different treatments were rinsed twice with deionized water. Samples for each treatment were dried for 24 h at 60°C, weighed and ashed in a 480°C oven for 16 h. After cooling, the ash was digested with 2 ml HNO_3 (65%) and heated to dryness. The sample was then dissolved in 5 ml 3 N HCl and brought to volume in a 25 ml volumetric flask using 0.1 N HCl. The Mn contents were determined by atomic absorption system (GVC 902, Australia).

RNA extraction

Total RNA of the various organs and F_2 flowers of different treatments were extracted using RNX-plus kit (RN7713C, CinnaGen, Iran) according to the manufacturer's instruction with slight optimization. Approximately 200 mg tissue from at least three different individual plants was used per sample and two RNA samples were extracted for each. The pellet was subsequently washed with ice-cold 75% ethanol, air-dried and dissolved in 40 µl of RNase–free water. The quality and concentration of RNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

cDNA synthesis

One µl of oligo (dT_{18}), 100 pmol, (K1621, Fermentas) was added to 5 µl total RNA and then reached 13 µl with RNase–free water. After being mixed, it was incubated at 70°C for 5 min and briefly chilled

Table 1. Primers used for semi-quantitative RT-PCR. *α-Tubulin* Accession number EC930869.

Gene	Primers 5' - 3'	Product (bp)
Limonene synthase	Forward: GATGATATTTACGATGTCTATGGTAC Reverse: GAATTGATTTCGGCACATCGCCTC	492
-Tubulin α	Forward: CAGCCAGATCTTCACGAGCTT Reverse: GTTCTCGCGCATTGACCATA	119

on ice. 4 µl of ×5 buffer (for ×1 final concentration) and 2 µl dNTP (10 mM) for 1 Mm final concentration were added to the mixture and the total volume was incubated at 37°C for 5 min. Afterwards, 1 unit RT-enzyme (K1621, Fermentas) was added and incubated at 42°C for 1 h. Inactivation of the reverse transcriptase accomplished by incubating the mixture at 70°C for 10 min. The cDNAs were then stored at -20°C until use.

Polymerase chain reaction (PCR) primer design

Since limonene synthase gene has not yet been sequenced in *C. cyminum*, degenerate primers were designed based on the highly conserved nucleotide sequences among several plants such as citrus, mentha and perilla genus. After obtaining the band by degenerate primers; by doing experiments with a few pairs of specific primers of citrus, mentha and perilla plants, eventually perilla specific primers were chosen (Table 1). The housekeeping gene α-tubulin was used as the standard for checking the quantity and quality of cDNA and/or RNA templates.

Semi- quantitative RT-PCR

1 µl of a four-fold dilution of the first-strand reaction (variable according to organs) was used as template for PCR amplification together with 1.3 µl of each primer and 12.5 µl Master Mix (PR8252C, CinnaGen, Iran) to a total volume of 25 µl. PCR amplification was performed using a thermocycler (Techne TC515, England) under the following conditions: 3 min at 94°C, followed by 28 or 35 cycles (for α-tubulin or LS respectively) of 45 s at 94 ^{O}C, 1 min at the annealing temperature at 54.8°C for α-tubulin and 42.5°C for LS, 1 min at 72°C and the final extension of 10 min at 72°C. After PCR, the samples were separated on 1.7% agarose gel and stained with ethidium bromide. Gel pictures were obtained using Gel-Doc Transilluminator (UVP Bioimaging system, USA). Densitometric evaluation of DNA bands was performed with the totalLab software version 1.10. All PCR analysis was repeated three times.

Verification of amplified products and sequencing

250 µl of PCR product was sequenced directly after 1% agarose gel fractionation and purification using the PCR gel extraction kit (K0513, fermentase) according to the manufacturer's instruction. Sequencing of purified PCR fragments was carried out by Genservice Company (England). LS gene sequencing was performed both for F_1 flower (<2 mm) and shoot.

Anatomical study

For anatomical observations, shoots and flowers (3-4 mm in size) were fixed in FAA. Cross-sections of the shoots and flowers were made with a Reichert sliding microtome and by hand–cutting. Sections were cleared in sodium hypochlorite and stained by carmine-vest (1% w/v in 50% ethanol) and methyl green (1% w/v, aqueous). They were mounted in gelatin and observed by optical microscope (Nikon YS 100).

Statistical analysis

Statistical analysis was performed using one–way analysis of variance (ANOVA). The F-ratio test indicated the existence of differences among treatments at $P \leq 0.05$. Different letters indicate significant differences among the treatments in a multiple range analysis for 95% confidence level. Each value is the mean ± S.E of three replicates.

RESULTS

Sequence analysis of the *C. cyminum* limonene synthase gene

Partial sequences of 492 bp amplicons for LS gene of cumin flower and shoot were sequenced and compared. BLAST analysis revealed perfect similarity between nucleotides according to ClustalW alignment. Figure 1A shows partial sequence of nucleotide and the deduced amino acid of LS gene of *C. cyminum* (492 bp). Nucleotide sequence of this gene revealed 68-88% identity to that of some other plants (Figure 1B). Partial sequence of limonene synthase gene of *C. cyminum* showed the highest homology of 88% to that of LS from *Mentha longifolia*. It also shared high homology with those of known LS from other plants (*Mentha spicata*, 85%; *Agastache rugosa,* 76%; *Perilla citriodora,* 71% and *Perilla frutescens*, 70%).

C. cyminum limonene synthase tissue-specific expression

To examine the expression patterns of the limonene synthase gene in *C. cyminum* organs the level of corresponding mRNA was monitored in different organs by semi- quantitative RT-PCR. As Figure 2 shows, there are large differences in expression levels of the LS gene in different organs. The highest expression was identified in the shoot and F_1 flower (<2 mm). Weaker expression was detected in F_2 flower (3-4 mm). No expression from

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TGAATTGATTTCGGCACATCGCCTCTCTTCAACTCATTCGATGAAGTTCCATAGTCGTCCGCTAGTCTAAA
 -   IDFGTSPLFNSFDEVP-SSASLK

      AATTTCTGACGCCAAGCGTACTATATCCGGAATGCCCTCTAAGAATTCCATTGCTTCTTTCTTGATTGGAT
                                ISDAKRTISGMPSKNSIASFLIG

TTGATGAGAAGATATAAGAATGAAGTACTATCGAAGGCCCCGTTATACTCACCAGTCCATTGCTCAAGTAC
FDEKI-E-STIEGPVILTSPLLKY

 TCCTGAAATGTAGGTTTATATCCACTGTAATACCACCGAGCCTCCACCATGTAGCGTTTCGACAATTCAAT
                                                    S-NVGLYPL-YHRASTM-RFDNSI

      CCATGCATTTCTTAGTTGCGGGAAAACATTGACATTCTGCTCGCTGAGGACGTCGAAAGCAATCTCGCTGG
                                HAFLSCGKTLTFCSLRTSKAISL

CGAAATTGTTAAGGGACAGAAAACATAATTTCATGTAGTGTGGAAGCTCCGTCATTGCATTTAAATCCCAT
AKLLRDRKHNFM-CGSSVIAFKSH

CTCTCGATAACACTTGTGAAGAGTTCAAGTTCATCCAGTGTACCATAGACATCGTAAATATCATCA
 L  S  I  T  L  V  K  S  S  S  S  S  S  V  P  -  T  S  -I   S  S
```

Figure 1A. Partial sequence of nucleotide and the deduced amino acid of limonene synthase gene of *Cuminum cyminum* (492 bp).

the F_3 (4-5 mm) and F_4 (>5 mm) flowers, roots and leaves was observed.

Expression pattern of limonene synthase gene in response to different concentrations of manganese

To characterize whether the *C. cyminum* limonene synthase gene expression is induced by different concentrations of manganese, the expression levels of this gene were investigated under various treatments. A semi–quantitative RT–PCR analysis was performed on total RNA from F_2 flowers (3-4 mm) under manganese treatments (0, 40, 80 and 160 ppm) as spray. Twice spray of 40, 80 and 160 ppm of manganese at PBAF40, PBAF80 and PBAF160 treatments increased the concentration of Mn approximately 1.2-1.3 fold in comparison with one spray only at blooming in PAF40, PAF80 and PAF160, respectively (Figure 3). The results revealed that exposure to 40 ppm of Mn decreased considerably limonene synthase gene expression both on T_1 (PBAF40) and on T_2 (PAF40) compared to controls (0 ppm of Mn) (Figure 4). The expression of LS gene was found to be up-regulated by 80 ppm of Mn especially at blooming phase. The highest concentration of Mn (160 ppm) significantly repressed the expression of LS gene. Such concentration of Mn caused the weakest expression of LS gene on both of late vegetative stages and blooming (PBAF160). Applying of 160 ppm concentration of Mn at blooming (PAF160) slightly enhanced the limonene synthase gene expression.

Existence of oil ducts in *C. cyminum* shoot

The results of this study confirmed the oil ducts location in flowers (fruits) of cumin (Figure 5A) as storage organs. But existence of oil ducts in cumin shoot was an unexpected and notable result (Figure 5B).

DISCUSSION

Expression of limonene synthase gene in different organs of *C. cyminum* L.

We reported here a partial cDNA sequence from LS gene in *Cuminum cyminum.* According to our knowledge, this is the first evidence for existence of limonene synthase gene in cumin. Biochemical studies had indicated the existence of limonene in fruits of cumin (Kan et al., 2007). In this study, we confirmed this assignment at the molecular level by studying the expression of LS gene in different organs of this plant. Results of this study revealed that LS gene expression is restricted to a short interval during fruit ontogeny in *C. cyminum*. The highest expression of the gene is in the youngest flowers (< 2 mm in size). These results are consistent with those of several previous investigations on plant terpene formation which revealed that high level of monoterpene biosynthesis in immature tissue. For example the biosynthesis of monoterpene in *S. officinalis* leaves (Croteau et al., 1981), *Majorana hortensis* leaves (Croteau, 1977b), *C. carvi* fruits (Boumeester et al., 1998),

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L13459.1        TGAACGAGGGTGGTGTTGATGGC------------------GACCTTTTAACAAGAATCG 755
AF175323.1      TGAACGAGGGTGGTGTTGATGGC------------------GACCTTTTAACAAGAATCG 764
AF233894.1      TCAATGACAAAACAATCGATGATGATGATGATGCAGACACTAATCTTATATCGTGTGTGC 785
D49368.1        TCAATGAGAAAACAATCGATGATGATGATGATGCAGACACTAATCTTATATCGTGTGTGC 760
AY055214.1      TGAAGGGTGATGAAATTGATGAC------------------AACCTTTTATCATCCATTA 816
cumin           ------------------------------------------------------------TGA 3
                                                                             *

L13459.1        CATATTCTTTGGACATCCCTCTTCATTGGAGGATTAAA--AGGCCAAATGCACCTGTGTG 813
AF175323.1      CATATTCTTTGGACATCCCACTTCATTGGAGGATTAAA--AGGCCAAATGCACCTGCGTG 822
AF233894.1      GCCACTCTTTGGACATCCCAATTCATTGGAGGATTCAA--AGGCCAAATGCAAGCTGGTG 843
D49368.1        GCCACTCTTTGGACATCCCAATTCATTGGAGGATTCAA--AGGCCAAATGCAAGCTGGTG 818
AY055214.1      GATATTCTTTAGAGATCCCAAACTATTGGAGTGTTGTA--AGGCCGAATGTAAGTGTGTG 874
cumin           ATTGATTTCGGCACATCGCCTCTCTTCAACTCATTCGATGAAGTTCCATAGTCGTCCGCT 63
                     * *       *****      *      **  *   * *    **       *

L13459.1        GATCGAATGGTATAGGAAGAGGCCCGACATGAATCCAGTAGTGTTGGAGCTTGCCATACT 873
AF175323.1      GATCGAATGGTATAGGAAGAGGCCCGACATGAATCCAGTAGTGTTGGAGCTTGCCATACT 882
AF233894.1      GATTGATGCATATAAGAGGAGAAGTCACATGAATCCACTTGTGTTGGAGCTTGCCAAACT 903
D49368.1        GATTGATGCCTATAAGAGGAGAAGTCACATGAATCCACTTGTGTTGGAGCTTGCCAAACT 878
AY055214.1      GATTGATGAATATAGGAAGAGATCAGACATGAATCCAGTGGTGTTGGAGCTCGCCATACT 934
cumin           AGTCTAAAAATTTCTGACGCCAAGCGTACTATATCCGG-AATGCC----CTCTAAGAATT 118
                  *  *     * *  ** *          *  ****     **       **     * *

L13459.1        CGACTTAAATATTGTTCAAGCACAATTTCAAGAAGAGCTCAAAGAATCCTTCAGGTGGTG 933
AF175323.1      CGACTTAAATATTGTTCAAGCACAGTTTCAAGAAGAGCTTAAAGAATCCTTCAGGTGGTG 942
AF233894.1      CGACTTAAATATTTTTCAAGCACAGTTTCAACAAGAACTCAAACAAGACTTAGGGTGGTG 963
D49368.1        CGACTTAAATATTTTTCAAGCACAGTTTCAACAAGAACTCAAACAAGACTTAGGGTGGTG 938
AY055214.1      GGACGCAAATATTGTTCAAGCACAATTGCAACTAGAACTAAAAGAATCACTAAGGTGGTG 994
cumin           CCA-TTGCTTCTTTCTTGATTG-GATTTGATGAGAAGATATAAGAA----TGAAGTACTA 172
                  *       * **  *  *      **  *     *    * ****    *  **  *

L13459.1        GAGAAATACTGGGTTTGTTGAGAAGCTGCCCTTCGCAAGGGATAGACTGGTGGAATGCTA 993
AF175323.1      GAGAAATACTGGGTTTGTTGAGAAGCTGCCCTTCGCAAGGGATAGACTGGTGGAATGCTA 1002
AF233894.1      GAAAAATACATGCCTTGCTGAGAAGCTCCCCTTTGCAAGGGATAGGCTTGTGGAATGCTA 1023
D49368.1        GAAAAATACATGCCTTGCTGAGAAGCTCCCCTTTGTAAGGGATAGGCTTGTGGAATGCTA 998
AY055214.1      GAGAAATACGTGCTTTGTGGAGAAGCTCCCGTTCGCGAGGGATAGGCTTATAGAGAGCTA 1054
cumin           TCGAAGGCCCCGTTATACTCACCAG-TCCATTGCTCAAGT-----ACTCCTGAAATGTAG 226
                   **    *  *   *       * **** *      **           *****

L13459.1        CTTTTGGAATACTGGGAT--CATCGAGCCACGTCAGCATGCAAGTGCAAGGATAATGATG 1051
AF175323.1      CTTTTGGAATACTGGGAT--CATCGAGCCACGTCAGCATGCAAGTGCAAGGATAATGATG 1060
AF233894.1      CTTTTGGTGTACTGGGAT--CATTCAGCCTCTTCAACATGAAAATGCAAGGGTAACGCTG 1081
D49368.1        CTTTTGGTGTACTGGGAT--CATTCAGCCTCTTCAACATGAAAATGCAAGGGTAACGCTG 1056
AY055214.1      CTTCTGGAGTACAGGGAT--GGTAGAGCCCCGTCAGCATGCAAACGCAAGGATAATTATG 1112
cumin           GTTTATATCCACTGTAATACCACCGAGCCTC--CACCATGTA-GCGTTTCGACAATTCAA 283
                 **        ** *  **       **** * ** **** *   *    *   **

L13459.1        GGCAAAGTCAACGCTCTGATTACGGTGATCGATGATATTTATGATGTCTATGGCACCTTA 1111
AF175323.1      GGCAAAGTCAACGCTCTGATTACGGTGATCGATGATATTTATGATGTCTATGGCACCTTA 1120
AF233894.1      GCCAAAGTTAACGCTCTAATAACCACGTTAGATGATATTTACGATGTCTATGGTACCTTA 1141
D49368.1        GCCAAAGTTAACGCTCTAATAACCACGTTAGATGATATTTACGATGTCTATGGTACCTTA 1116
AY055214.1      GCCAAAGTTATCGCTCTTATAACAGTCATGGATGATATTTACGATGTCTATGGTACCTTA 1172
cumin           TCCATG--CATTTCTTAGTTGCGGGAAAACATTGACATTCTGCTCGCTGAGGACGTCGAA 341
                   **    *   **    *             *** ***     *   * *    *  *

L13459.1        GAAGAACTCGAACAATTCACTGACCTCATTCGAAGATGGGATATAAACTCAATCGACCAA 1171
AF175323.1      GAAGAACTCGAACAATTCACTGACCTCATTCGAAGATGGGATATAAACTCAATCGACCAA 1180
AF233894.1      GAAGAACTCGAGCTATTCACCGAGGCGATTCGGAGATGGGATGTTAGTTCAATTGACCAT 1201
D49368.1        GAAGAACTCGAGCTATTCACCGAGGCGATTCGGAGATGGGATGTTAGTTCAATTGACCAT 1176
AY055214.1      GAAGAACTCGAACAATTCACAGAGGCCTTTCGAAGATGGGATGTGAGTTCAATCGACCAA 1232
cumin           AGCAATCTCGCTGGCGAAATTGTTAAGGGACAGAAAACATAATTTCATGTAGTGTGGAAG 401
                    * ****         *  *        * * *     * *      * *      *

L13459.1        CTTCCCGATTACATGCAACTGTGCTTTCTTGCACTCAACAACTTCGTCGATGATACAT-C 1230
AF175323.1      CTTCCCGATTACATGCAACTGTGCTTTCTTGCACTCAACAACTTCGTCGATGATACAT-C 1239
AF233894.1      CTTCCTAATTACATGCAACTCTGTTTTCTTGCACTCAACAATTTCGTCGACGACACAG-C 1260
D49368.1        CTTCCTAATTACATGCAACTCTGTTTTCTTGCACTCAACAATTTCGTGGACGACACAG-C 1235
AY055214.1      CTTCCTACTTATATGCAACTGTGTTTTCTTGCAATCAACAACTTTGTGGACGACACGG-C 1291
cumin           CTCCGTCATTGCATTTAAATCCCATCTCTCGA---TAACACTTGTGAAGAGTTCAAGTTC 458
                 ** *   **   ** ** *       *****    ****   * *   **    *   *

L13459.1        GTACGATGTTATGAAGGAGAAAGGCGTCAACGTTATACCCTACCTGCGGCAATCGTGGGT 1290
AF175323.1      GTACGATGTTATGAAGGAGAAAGGCGTCAACGTTATACCCTACCTGCGGCAATCGTGGGT 1299
AF233894.1      TTATGATGTCATGAAAGAGAAAGATATCAACATCATCCCGTATCTACGAAAATCGTGGTT 1320
D49368.1        TTATGATGTCATGAAAGAGAAAGATATTAACATCATCCCGTATCTACGCAAATCGTGGTT 1295
AY055214.1      ATACAATGTTCTGAAAGAGAGTGGTGTCAATGTTATGACATATCTCCGAAAATCGTGGGT 1351
cumin           ATCCAGTGT-ACCATAGACATCGTAAATATCATCA------------------------- 492
                 *     ***    * ** *  *     *   * *
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Figure 1B. Alignment of nucleotide sequence of *Cuminum cyminum* limonene synthase. Accession numbers: L13459.1, *Mentha spicata*; AF175323.1, *Mentha longifolia*; AF233894.1, *Perilla citriodora*; D49368.1*, perilla frutescens*; AY055214.1: *Agastache rugosa*; cumin, *Cuminum cyminum*.

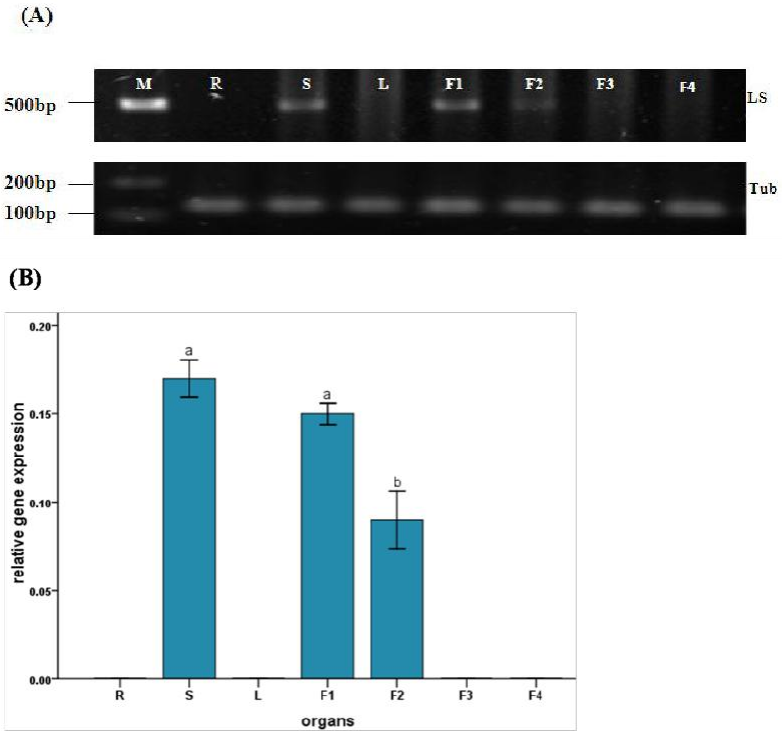

Figure 2. Semi-quantitative RT-PCR showing differential expression of limonene synthase gene in organs of *C. cyminum*. LS, limonene synthase (492 bp); Tub, tubulin (119 bp); M, 100 bp DNA ladder; R, root; S, shoot; L, leaf; F_1, flower < 2 mm; F_2, flower 3-4 mm; F_3, flower 4-5 mm; F_4, flower > 5 mm. The tubulin gene was used as housekeeping. (**A**) Agarose gel electrophoresis of SQ-RT-PCR. (**B**) Densitometric analysis of gel bands.The different letters above the bars are significantly different from each other at $P \leq 0.05$.

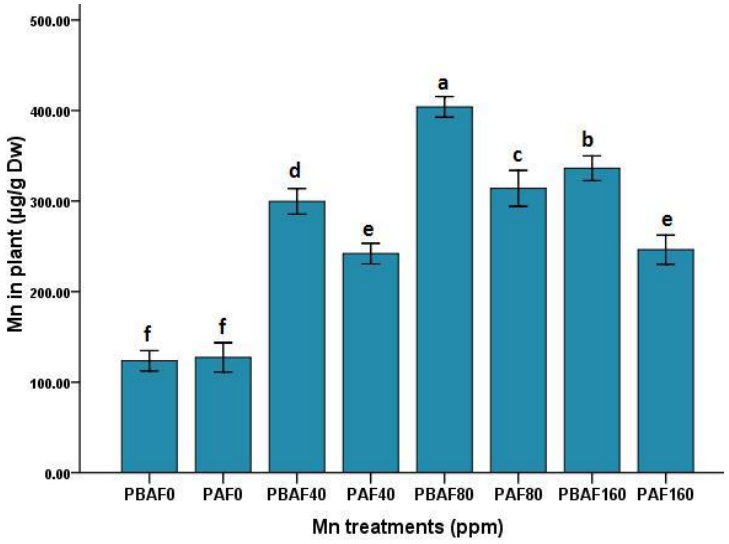

Figure 3. Effects of different tratments on Mn content of the aerial parts of *C. cyminum*. treatments: PBAF0, PBAF40, PBAF80 and PBAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese respectively, at the last stages of vegetative phase and blooming; PAF0, PAF40, PAF80 and PAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese respectively, only at blooming. The different letters above the bars are significantly different from each other at $P \leq 0.05$.

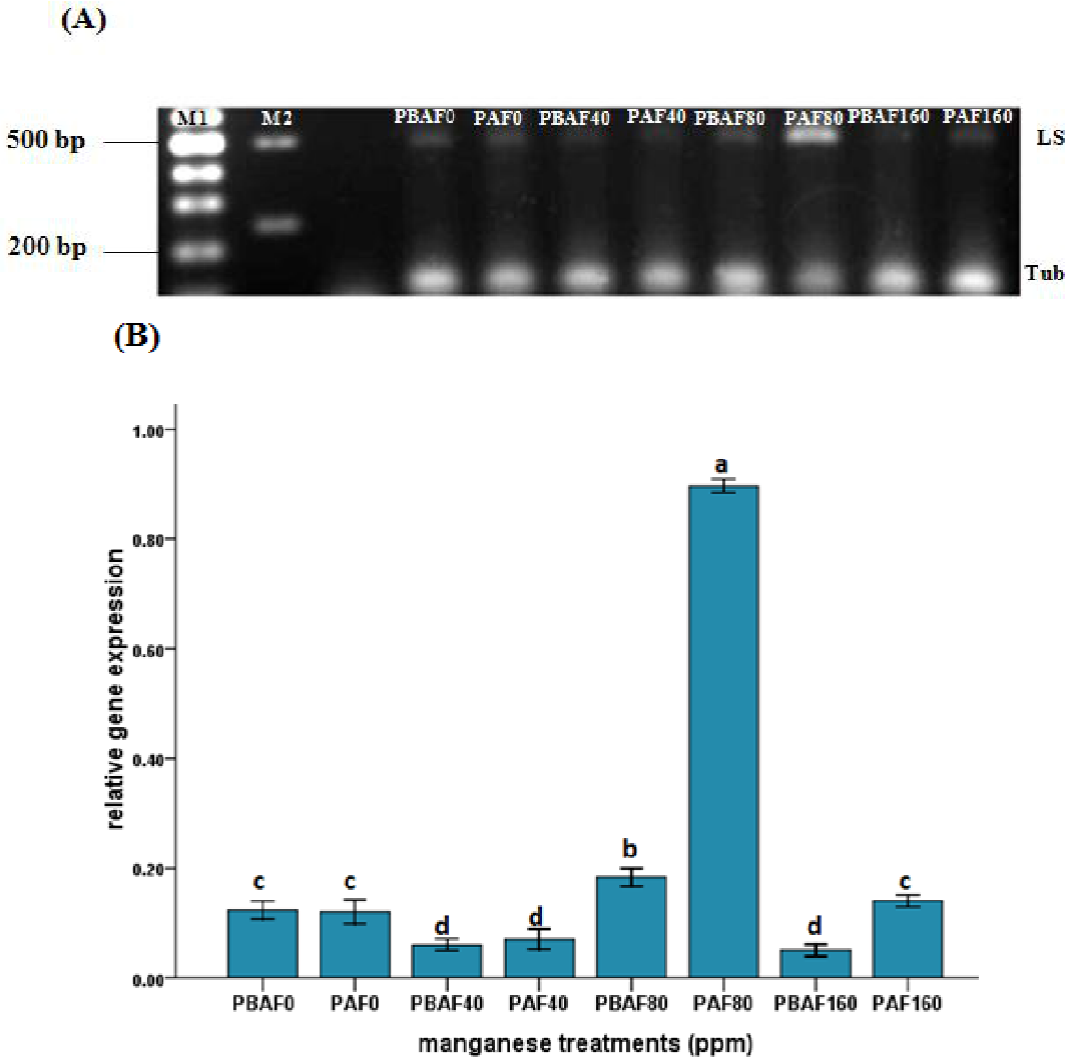

Figure 4. Effects of different concentration of manganese on the limonene synthase gene expression of *C. cyminum*. LS, limonene synthase (492 bp); Tub, tubulin (119 bp); M_1, 100 bp DNA ladder; M_2, 1000 bp DNA ladder; treatments: PBAF0, PBAF40, PBAF80 and PBAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese respectively, at late stages of vegetative phase and blooming; PAF0, PAF40, PAF80 and PAF160 sprayed with distilled water and the concentrations of 40, 80 and 160 ppm of manganese respectively, only at blooming. (A) Agarose gel electrophoresis of SQ-RT-PCR. (B) Densitometric analysis of gel bands. The different letters above the bars are significantly different from each other at $P \leq 0.05$.

C. flexuosus blades (Singh et al., 1989) and maritime pine (*Pinus pinaste*r) foliage (Bernard-Dagan, 1982) is restricted to a short interval during organ ontogeny. Accumulation of monoterpenes is confined to the early stages of fruit development so that young fruits contain high concentrations of limonene in *Carum carvi* L. (Boumeester et al., 1998). Similar to caraway it appears possible that accumulation of limonene in fruits of cumin is a developmentally regulated process. During the early stages of caraway fruit development there is an abundant accumulation of limonene and limonene synthase enzyme exhibits high levels of activity (Boumeester et al., 1998). Similar changes in terpenoid accumulation patterns occur during plant development in some other plant species including dill Porter et al., 1983) and peppermint (Voirin and Bayet, 1996). Monoterpenes are believed to function principally in ecological roles, serving as herbivore-feeding deterrents, antifungal defense and attractants for pollinators (Langenheim, 1994). It seems likely that a sufficient amount of free substrate is available or can be made available, particularly in young flowers. In flowers, the GPP pool is large and is not limiting terpene production (Lucker et al., 2004). This pool of GPP in young tissues may benefit the plant by allowing the rapid production of monoterpenes to repel herbivores or attract predators of the herbivores and thus protect

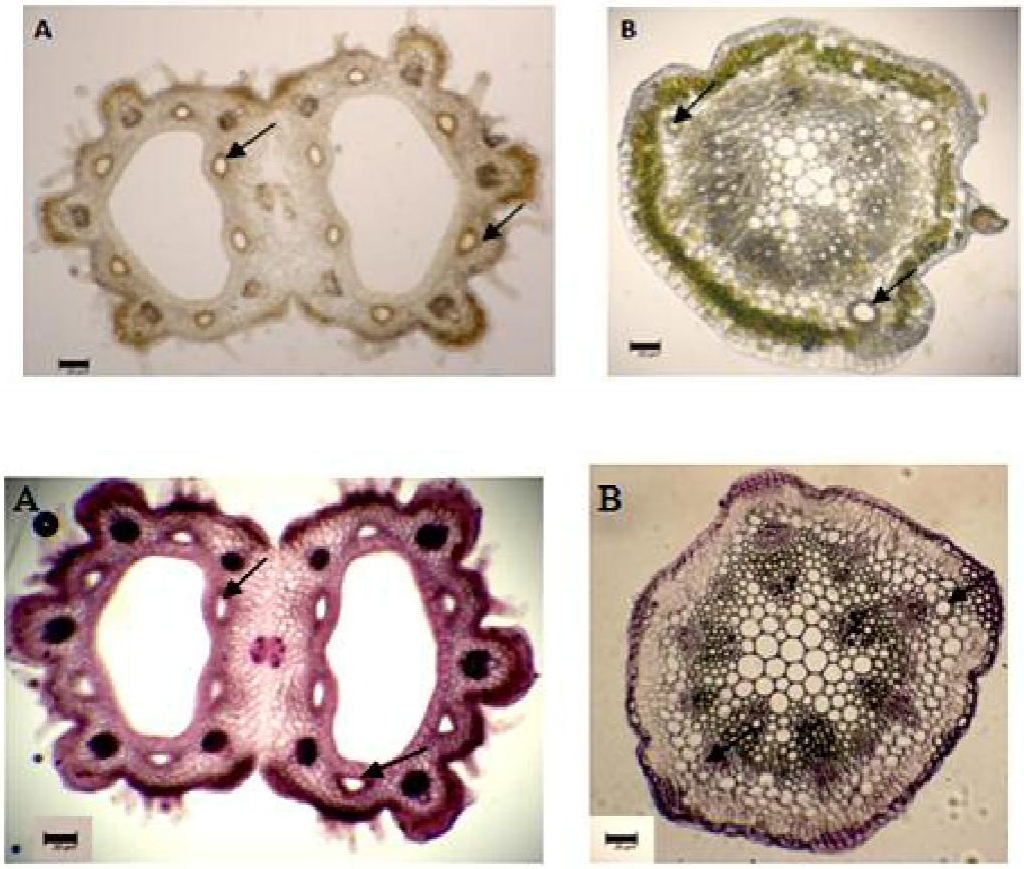

Figure 5. Cross sections from flower 3-4 mm in size (A) and shoot (B) of *Cuminum cyminum*. Arrows indicate oil ducts (Scale bar 20 µm).

new growth. Results of this study revealed that limonene synthase gene is not expressed in larger flowers (>4 mm). Boumeester et al. (1998) showed that, amount of limonene and limonene synthase activity declined to low levels at later stages of caraway fruit development. Also, it has been demonstrated that limonene serves as the common olefinic precursor of the essential oil terpenes such as peppermint and spearmint (Kjonas and Croteau, 1983) through a series of secondary, largely redox, transformations (Croteau and Gershenzon, 1994). In peppermint, during leaves development, the percentage of limonene drops and the percentage of oxygenated products menthone and menthol increases (Voirin and Bayet, 1996; Brun et al., 1991).

Termination of the expression of LS gene in larger flowers is probably accompanied by the expression of other genes such as limonene-6-hydroxylase for conversion of limonene as precursor to storage derivative compounds in ripened fruits (Boumeester et al., 1998). Accordingly, expression of genes participating in such metabolic pathway should be considered. It seems that terpenoids such as limonene play protective role in this organ or participate for production and transformation of such material onto flowers (fruits).

Manganese changes limonene synthase gene expression in *C. cyminum*

Manganese-dependent gene expression is not fully understood in eukaryotic organisms. In turn, the homeostasis of this metal appears to involve a complex network of proteins (Gutierrez et al., 2008). It has been suggested that Mn^{+2} might induce the expression of manganese–dependent peroxidase through metal-responsive elements (MREs) located in the promoters of the corresponding genes [8]. MREs have been identified in animals and plants as the target site of transcription factors responding to cadmium, zinc and copper (Thiele, 1992). Mn^{+2} have a transcriptional role which would likely imply a putative transcription factor (Ma et al., 2004). Previous results have reported that application of micronutrients, as supplements to macroelements, leads to significant effects on herb yield and oil contents of many plants (Wahab and Hornok, 1983; Sharma et al., 1980; El-Sawi and Mohamed, 2002).

It is well known that terpenoids are the major components of most plants essential oils (Guenther, 1950) and limonene is precursor for most of these compounds and their derivatives. In the isoprenoid biosynthetic pathway

there are several enzymatic reactions that require or can use Mn as a cofactor (Wilkinson and Ohki, 1988). Probably the effects of Mn on LS gene expression might be due to enzyme cofactor (Coates et al., 1997) or transcription factor (Ma et al., 2004) roles for this element. Enzyme activity usually requires a specific amount of a metal (Mn) that any change in its concentration can cause repression (Wilkinson and Ohki, 1988). It can thus be assumed that 80 ppm concentration of manganese can be considered as optimum for limonene synthase gene expression of *C. cyminum* specially at blooming. Flowers (fruits) are the organs of production and storage of secondary metabolites containing terpenoids in cumin. Accordingly the effect of Mn (80 ppm) on blooming is reasonable. Results of the present research project revealed that not only concentration of 40 ppm of Mn at both methods but also 160 ppm at T_1 method decreased limonene synthase gene expression. Decrease in transcription levels may be the result from a decreased stability of transcripts (Fitzgerald et al., 2008). Usually biphasic responses of plants to increasing Mn concentration were observed; efficient adaptation and toxic heavy metals accumulation lead to oxidative stress (Lidon and Teixeira, 2000). On this basis, on the one hand 40 ppm concentration can be considered as deficiency concentration for LS gene expression and on the other hand 'efficient adaptation' leads to oxidative stress. 160 ppm of Mn repressed LS gene probably due to effects on LS enzyme with negative feedback or oxidative stress. Oxidative stress caused by Mn can result in damage to proteins (Demirevska-Kepova et al., 2004) such as enzymes (limonene synthase) and transcription factors and so can cause repression of related gene expression. Peroxidase induction has been observed as a general response of higher plants to toxic amounts of heavy metals (Van Assche and Clijsters, 1990).

Existence of oil ducts in flower and shoot of cumin

Plant volatile oils are associated usually with secretory structures; resin ducts, lysigenous glands and glandular trichomes (Schnepf, 1974; Dell and McComb, 1978a; Dell and McComb, 1978b; Fahn, 1979). Secretory ducts are tubular structures, often branching to create a network extending from the roots through the stem to the leaves, flowers and fruits. They are composed of an epithelium which surrounds a central cavity. The Apiaceae is a family represented by numerous members bearing oil ducts. Despite the commercial and pharmaceutical significance of the essential oils produced by many Apiaceae, the number of investigations referring to the structure of the oil ducts is limited (Bosabalidis, 1996). Existence of oil ducts in cumin fruits in this study is concensus with many biochemical studies in this regard (Boumeester et al., 1998; Kan et al., 2007). Besides existence of oil duct in cumin shoot is concensus with Bosabalidis anatomical studies (1996) in celery (*Apium graveolens* L.) (Bosabalidis, 1996). However, Due to the fact that shoot is not a storage organ for secondary metabolites such as terpenoids and their derivatives in cumin, limonene synthase gene expression and existence of oil ducts in cumin shoot can be remarkable results for more studies. In future, further work such as measuring LS enzyme activity, expression of other related genes and produced materials could be accounted in order to elucidate the correlation between limonene synthase gene expression in different organs, developmental stages and their relation with manganese concentration. Moreover, analysis of the promoter sequence of LS gene can yield information of consensus motifs which might identify common functional regulatory elements to metals such as Mn that have not yet been defined.

REFERENCES

Amin GH (2000). Cumin. In: Peter, K.V. (ed.), Handbook of Herbs and Spices. Woodhead Publishing, Cambridge, UK, pp. 164-167.

Aruna K, Rukkumani R, Suresh Varma P, Venugopal MP (2005). Therapeutic role of *Cuminum cyminum* on ethanol and thermally oxidized sunflower oil induced toxicity. Phytother. Res., 5: 416-421.

Aslam M, Jafri MA, Javed K, Singh S (2003). Evaluation of antidiabetic drugs from plant sources. In: Singh S, Govil JN, Singh VK. (eds) Recent Progress in Medicinal Plants. Studium Press, Houston, Texas. Phytochem. Pharmacol., 2: 105.

Berna A, Bernier F (1999). Regulation by biotc and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H_2O_2 producing enzyme. Plant Mol. Biol., 39: 539- 549.

Bernard-Dagan C, Pauly G, Marpeau A, Gleizes M, Carde JP, Baradat P (1982). Control and compartmentation of terpene biosynthesis in leaves of *pinus pinaster*. Physiol. Veg., 20: 775-795.

Bosabalidis AM (1996). Ontogenesis, Ultrastructure and Morphometry of the Petiole Oil Ducts of Celery (*Apium graveolens* L.). Flavour Frag. J., 11: 269-274.

Boumeester HJ, Gershenzon J, Konings MCJM, Croteau R (1998). Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway: I. Demonstration of enzyme activities and their changes with development. Plant Physiol., 117: 901-912.

Brun N, Colson M, Perrin A, Voirin B (1991). Chemical and morphological studies of the effects of aging on monoterpene composition in *menthe* × *piperita* leaves. Can J. Bot., 69: 2271-2278.

Buckingham J (1998). Dictionary of natural products on CD-ROM, version 6.1. Chapman and Hall, London.

Coates RM, Elmore CS, Croteau RB, Williams DC, Morimoto H, Williams PG (1997). Stereochemistry of the methyl methylene elimination in the enzyme-catalysed cyclization of geranyl diphosphate to (4S)-limonene. Chem. Commun., 21: 2079-2080.

Croteau R, Felton M, Karp F, Kjonaas R (1981). Relationship of camphor biosynthesis to leaf development in sage (*Salvia officinalis*). Plant Physiol., 67: 820-824.

Croteau R, Gershenzon J (1994). Genetic control of monoterpene biosynthesis in mints (*Mentha: Lamiaceae*). Recent Adv. Phytochem., 28: 193-229.

Croteau R (1977b). Site of monoterpene biosynthesis in *Majorana hortensis* leaves. Plant Physiol., 59: 519-520.

Crowley JD, Traynor DA, Weatherburn DC (2000). Enzymes and proteins containing manganese: an overview. Met Ions Biol. Syst., 37: 209-278.

Culotta VC, Yang M, Hall MD (2005). Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*. Eukaryot Cell, 4:1159-1165.

Dell B, McComb AJ (1978a) Plant resins and their formation, secretion and possible functions. In: Woolhouse HW, ed. Advances in Botanical Res., 6: 78-316.

Dell B, McComb AJ (1978b). Glandular hair formation and resin secretion in *Eremophila fraseri*. F. Meull (Myoporaceae). *Protoplasma*, 92: 71-86.

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Hölzer R, Feller U (2004). Biochemical changes in barley plants after excessive supply of copper and manganese. Environ. Exp. Bot., 52: 253-266.

El-Ghadban EAE (1994). The effect of some trace elements on growth and oil yield of spearmint (*Menth viridis* L.). M. Sc. Thesis, Fac. Agric., Cario Univ., Egypt.

El-Khateeb MA, Farahat M, Boselah NAE (1994). The effect of trace elements on growth, yield and chemical constituents of rue plants (*Ruta graveolens* L.). Egypt. J. Appl. Sci., 7: 75-95.

El-Sawi SA, Mohamed MA (2002). Cumin herb as a new source of essential oils and its response to foliar spray with some micro-elements. Food Chem., 77: 75-80.

Fahn A (1979). Secretory tissues in plants. New York: Academic Press, pp. 158-222.

Fitzgerald TL, Waters DLE, Henry RJ (2008). The effect of salt on betaine aldehyde dehydrogenase transcript levels and 2-acetyl-1-pyrroline concentration in fragrant and non-fragrant rice (*Oryza sativa*). Plant Sci., 175: 539-546.

Gagandeep S, Dhanalakshmi E, Mendiz A, Rao R, Kale RK (2003). Chemopreventive effects of *Cuminum cyminum* in chemically induced forestomach and uterine cervix tumors in murine model systems. Nutr. Cancer, 2: 171-180.

Gershenzon J, Croteau R (1990). Regulation of monoterpene biosynthesis in higher plants. Recent Adv. Phytochem., 24: 99-160.

Guenther E (1950). The essential oils. D. Van Nostrand Company, Inc., New York.

Gutierrez M, Rojas LA, Mancilla-Villalobos R, Seelenfreund D, Vicuna R, Lobos S (2008). Analysis of manganese-regulated gene expression in the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Curr. Genet., 54:163-173.

Hagen G, Uhrhammer N, Guilfoyle TJ (1988). Regulation of expression of an auxin –induced soybean sequence by cadmium. J. Biol. Chem., 263: 6442-6446.

Janahmadi M, Niazi F, Danyali S, Kamalinejad M (2006). Effects of the fruit essential oil of *Cuminum cyminum* Linn. (Apiaceae) on pentylenetetrazol-induced epileptiform activity in F1 neurones of Helix aspersa. J. Ethnopharmacol., 1-2: 278-282.

Kan Y, Kartal M, Ozek T, Aslan S, Baser KHC (2007). Composition of essential oil of *cuminum cyminum* L. according to harvesting times. Turkish J. Pharm. Sci., 1: 25-29.

Kassem AHM (2002). Effect of planting distances and some trace elements on rosemary plant. Ph. D. Thesis, Fac. Agric., Cario Univ., Egypt.

Keen CL, Ensunsa JL, Clegg MS (2000). Manganese metabolism in animals and humans including the toxicity of manganese. Met Ions Biol. Syst., 37: 89-121.

Kjonas R, Croteau R (1983). Demonstration that limonene is the first cyclic intermediate in the biosynthesis of oxygenated p-menthane monoterpenes in *Mentha piperita* and other *Mentha* species. Arch Biochem. Biophys., 220: 79-89.

Langenheim JH (1994). Higher plant terpenoids: a phytocentric overview of their ecological roles. J. Chem. Ecol., 20: 1223–1280.

Lescure AM, Proudhon D, Pesey H, Raglnd M, Theil EC, Briat JF (1991). Ferritin gene transcription is regulated by iron in soybean cell cultures. Proc Natl Acad. Sci. USA, 88: 8222- 8226.

Lidon FC, Teixeira MG (2000). Oxy radicals production and control in the chloroplast of Mn-treated rice. Plant Sci., 152: 7-15.

Lucker J, Schwab W, Hautum BV, Blaas J, Plas LHW, Bouwmeester HJ, Verhoeven HA (2004). Increased and Altered Fragrance of Tobacco Plants after Metabolic Engineering Using Three Monoterpene Synthases from Lemon. Plant Physiol., 134: 510-519.

Ma B, Mayfield MB, Godfrey BJ, Gold MH (2004). Novel promoter sequence required for manganese regulation of manganese peroxidase isozyme 1 gene expression in *Phanerochaete chrysosporium*. Eukaryot Cell, 3: 579-588.

Mahmoud SS, Croteau RB (2002). Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends Plant Sci., 7: 366-373.

Marschner H (1995). Mineral nutrition of higher plants. Academic Press. London, UK, p. 889.

Munoz-Bertomeu J, Ros R, Arrillaga I, Segura J (2008). Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. Metab. Eng., 10: 166-177.

Nandi RP, Chatterjee SK (1991). Improved cultivation and distillation methods, followed by citronella plantations of Darjeeling hills. Indian Perfumer, 35: 80-85.

Porter NG, Shaw ML, Shaw GJ, Ellingham PJ (1983). Content and composition of dill herb oil in the whole plant and the different plant parts during crop development. New Zealand J. Agric. Res., 26: 119-127.

Schnepf E (1974). Gland cells. In: Robards AW, ed. Dynamic aspects of plant ultrastructure. London: McGreaw-Hill, pp. 331-357.

Sharma SN, Singh R, Tripathi S (1980). Effect of NPK and micronutrients on herb, oil and menthol yield of Japanese mint. Indian J. Agron., 2: 279-281.

Singh N, Luthra R, Sangwan RS (1989). Effect of leaf position and age on the essential oil quantity and quality in lemongrass (*Cymbopogon flexuosus*). Planta Med., 55: 254-256.

Spices Board Stastistics (2006). Spices Board, Kochi, India.

Swaefy HMFA (2002). Physiological studies on *Trachyspermum ammi* L. (*Carum copticum* Benth) plant. Ph. D. Thesis, Fac. Agric., Cario Univ., Egypt.

Thiele DJ (1992). Metal-regulated transcription in eukaryotes. Nucleic Acids Res., 20: 1183-1191.

Van Assche F, Clijsters H (1990). Effects of the metals on enzyme activity in plants. Plant Cell Environ., 3: 195-206.

Voirin B, Bayet C (1996). Developmental changes in the monoterpene composition of *Menthe* × *Piperita* leaves from individual peltate trichomes. Phytochemistry, 43: 537-580.

Wahab ASA, Hornok L (1983). Effect of NPK fertilization on yield and oil content of *Ocimum basilicum*. Kerteszeti Egypten Kozlemenye, 13: 66.

Weiss EA (2002). Umbelliferae. In: Spice Crops. CAB International, Wallingford, UK, pp. 261-268.

Wilkinson RE, Ohki K (1988). Influence of Manganese Deficiency and Toxicity on Isoprenoid Syntheses. Plant Physiol., 87: 841-846.