SUPPLEMENTARY MATERIAL

Effect of jasmonic acid on major terpenes and density of glandular trichomes in *Lippia graveolens* Kunth (Verbenaceae)

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The effect of exogenous application of jasmonic acid (JA) on the concentration of main terpenes and density of glandular trichomes was investigated in the Mexican oregano, propagated from seeds from 3 localities. JA 1 mM was applied locally and to the whole plant. JA locally applied increased the number of trichomes, with a mean of 20 trichomes more with respect to the controls in plants from Tecomavaca and Zapotitlán Salinas, and significantly increased the thymol concentration by 185% systemically and 255% locally, compared to the control. JA applied to the whole plant decreased the number of trichomes and increased the concentration of caryophyllene from 0.79 to 1.7 mg g⁻¹, and α-caryophyllene from 0.3 to 0.8 mg g⁻¹ in plants from San Rafael with reference to water control. The results suggest a plasticity of morphologic and phytochemical responses, and a potential use of JA to improve phenolic monoterpenes and sesquiterpenes production.

Keywords: Oregano, Elicitor, Trichomes, Thymol, Carvacrol, Hormone
Experimental

Plant Material

Seeds were collected from San Rafael (Puebla) at 952-989 m a.s.l.; Zapotitlán Salinas (Puebla) at 1433 m a.s.l., and Santa María Tecomavaca (Oaxaca) at 600 m a.s.l. located in the Tehuacán-Cuicatlán Valley. San Rafael has a climate which corresponds to dry or arid with summer rains (average annual rainfall of 395 mm) and a mean temperature of 22°C. The vegetation is a thorn scrub forest where species Bursera morelensis, Bursera aptera, Pachycereus weberi, Opuntia puberula and Ceiba parvifolia predominate (Casas et al. 2001; Canales et al. 2005). The soil of the area is underdeveloped which can be regosol or xerosol. Zapotitlán Salinas has an annual main temperature of 21°C and an annual rainfall from 400 to 450 mm. Edaphologically, soils are often stony with high levels of salinity (López-Galindo et al. 2003). The plant community is composed by mezquital, Prosopis laevigata; thorn scrub forest (Mimosa luisana, Acacia farnesiana, Cordia curassavica and Fouquieria formosa); tectehera (defined by dominance of Neobuxbaumia tetetzo); low dry deciduous forest (Myrtilocactus geometrizans, Bursera schlechtendalii and Bursera aptera) and cardonal (predominance of Cephalocereus columna-trajani) (Osorio-Beristain et al. 1996). In Santa María Tecomavaca the annual main temperature is 24.5°C and the annual rainfall is between 500 and 600 mm with an arid or semi-arid climate. The soils vary among fluvisol (origin from materials carrying by water), regosol (often shallow and stony) and luvisol (high clay content and susceptible to erosion). The predominant type of vegetation is low dry deciduous forest with species Pachycereus marginatus, Cephalocereus columnna-trajani, Mitrocereus fulviceps, Plumeria rubra, Bursera morelensis, B. schlechtendalii, B. submoniliformis, B. aptera, Acacia angustissima, Stenocereus stellatus, Mammillaria sphaellata, M. supertexta, M. haageana, Ferocactus flavovirens and F. recurvus (Grupo Mesófilo 2001). The 3 locations are relatively close, about 115 Km between the most distant, and climatically similar, however there are critical environmental factors such as air humidity or soil salinity that could affect the composition of the metabolites in plant populations. On the other hand, ethnobotanical studies have shown the medicinal importance of this species although its use varies according to the population: people in Zapotitlán Salinas use L. graveolens to treat gastrointestinal disorders (Hernández et al. 2003), while in San Rafael it is used by women for treatment of abdominal pain and induction of menstruation and delivery (Canales et al. 2005) which may be associated with a differential phytochemical profile.

Voucher specimens were deposited at the IZTA Herbarium in FES Iztacala (UNAM, Mexico) under registration numbers Izt 42699, Izt 42780 and Izt 2686 for specimens from San Rafael, Zapotitlán Salinas and Santa María Tecomavaca, respectively.

The seeds were disinfected using 1% NaOCl for 5 min, and then imbibed for 24 h in distilled water prior to being sown in 1% bacteriological agar under a constant fluorescent light of 81 µmol m⁻² s⁻¹ at 25°C, with maximum relative humidity of 82% and a photoperiod of 12L/12D.
Seedlings with 4 true leaves were transferred to 29.7 cm diameter pots with a humus:agrolite (1:1, v/v) substrate. Hoagland solution was added to the substrate to 80% of field capacity in order to avoid nutritional deficiencies. The pots were covered with a transparent bag to avoid contact with any potential herbivore. Physiological parameters, including photosynthesis, stomatal conductance and transpiration were recorded in seedlings with and without the transparent bag covering with no significant differences found between the groups (Table S1). Data were measured using a Li-Cor 6400 (Lincoln, Nebraska, USA) analyzer, operating at a leaf temperature of 25 °C and 1500 µmol m⁻²s⁻¹ of photosynthetic photon flux. Readings were taken on the apical leaf of five plants per group. Seedlings were kept in the growth chamber during treatments under the same conditions described above.

**Jasmonic Acid Treatments**

Four-month old plants of similar size, before flowering, were divided into two groups (locally treated and whole plant treated with JA). Each group was further divided into three subgroups of 15 plants each: control (distilled water), treatment (JA 1 mM dissolved in acetone 1%) and solvent control (distilled water and acetone 1%). Locally treated plants were subjected to one application of 120 µL of JA on the adaxial side of two not fully expanded young leaves of the second apical node of each plant with a micropipette. Treated leaves (only the leaf blade) were collected after 48 h. The same leaves were harvested in control plants which were in the same developmental stage to control ontogenic variation in density of trichomes. To monoterpenoid analysis, was collected also a mature leaf from the second node in order to determine systemic effect of JA on metabolites (Babst et al. 2009). Whole plant treatment consisted of the application of 1.5 mL of JA sprayed on the whole plant, on the adaxial and abaxial sides using a hand sprayer. From each replicate, the youngest new leaf formed following the application of treatments of the first basal node (average size of 14.1±0.5 mm length and 6.3±0.5 mm width) and a mature leaf from the second basal node (average size of 23.2±0.8 mm length and 10.3±0.2 mm width) were sampled eight days after treatment. As in the local treatment, the same leaves were collected in control plants. Samples taken for monoterpenoid analysis and for SEM were taken simultaneously.

**Scanning Electron Microscopy Imaging of Glandular Trichomes**

Longitudinal sections of leaf tissue measuring 0.5 cm² were mounted on aluminium stabs. The sections were examined and photographed at an accelerating voltage of 10 kV with a scanning electron microscope JSM-6380LV (Jeol Ltd.). Three images of each replicate, from the apical and basal regions of each leaf, were obtained at 100X magnification. We counted the glandular trichomes on the adaxial and abaxial surfaces with the Image J software V. 1.46 from images taken from the central part of the leaf.

**Identification and Quantification of Monoterpenes**
Leaf samples were pulverized in liquid nitrogen, homogenized with HPLC-grade hexane (Honeywell) and centrifuged at 15,000×g for 3 min. 4-isopropylphenol (Sigma-Aldrich) was used as an internal standard (Abu-Lafi et al. 2008). One μL of supernatant was injected by split injection (80:1) into an Agilent Technologies chromatograph model 6850 with an Agilent 19091s-433E HP-5 capillary column (5% phenyl methyl siloxane; 30 m x 0.25 mm, 0.25 μm). The injector temperature was 250°C. Helium was used as a carrier gas (20 mL min⁻¹) and the oven temperature program maintained at 70°C for 2 min, followed by a ramp of 20°C min⁻¹ to 230°C and then 8°C min⁻¹ to 280°C (5 min). Detection was via an Agilent Technologies mass spectrometer 5975C VL MSD with an ionization voltage of 70 eV. Carvacrol and thymol were identified using standards (Sigma-Aldrich). Other monoterpenes were identified based on spectral comparison with the NIST08 database.

**Statistical Analyses**

The results are reported as mean values ± SE. We used a two-way analysis of variance (ANOVA) to compare the variables among treatments, and to compare the effect of leaf region (apical and basal) and plant node based using the GenStat 11 statistical package. The significance of differences among treatments was tested using LSD Fischer test $P \leq 0.05$. Student’s $t$ test was used ($P \leq 0.05$) to compare between groups in 2-group comparisons (adaxial/abaxial, apical/basal side, node 1/node 2). A principal component analysis was performed using the R program version 3.0.2.
Table S1. Physiological parameters in seedlings of *L. graveolens* with and without the transparent bag covering. Data recorded with Infrared Gas Analizer Li-Cor 6400 using 1500 µmol m$^{-2}$s$^{-1}$ of photosynthetically active radiation PAR and at 25 °C. P values correspond to the comparison of means between the two groups (Student’s *t* <0.05). Values are means ± SE based on 5 replicates.

| Parameter                                      | With bag     | Without bag  | P value |
|-----------------------------------------------|--------------|--------------|---------|
| Assimilation rate of CO$_2$ (µmol CO$_2$ m$^{-2}$s$^{-1}$) | 1.96±0.19    | 1.48±0.14    | 0.092   |
| Stomatal conductance (mol H$_2$O m$^{-2}$s$^{-1}$)       | 0.013±0.002  | 0.025±0.013  | 0.936   |
| Transpiration (mmol H$_2$O m$^{-2}$s$^{-1}$)             | 0.43±0.07    | 0.55±0.06    | 0.078   |
Table S2. Number of glandular trichomes per mm$^2$ on leaves of *L. graveolens* grown from seeds of 3 localities: San Rafael (SR), Tecomavaca (T) and Zapotitlán Salinas (Z), treated with 1.5 mL of 1mM jasmonic acid (JA), 1.5 mL of water (W) or 1.5 mL of distilled water + acetone 1% (AC) sprayed to the whole plant. For a given treatment, bars that share letters are not significantly different (LSD >0.05), P$^1$ values correspond to the comparison between adaxial and abaxial (Student’s t <0.05) density, and P$^2$ values corresponds to the comparison among treatments. Values are means ± SE based on 5 replicates.

| Node      | Node 1: Young leaves | Node 2: Mature leaves |
|-----------|----------------------|-----------------------|
|           | Apical | Basal | P$^1$ | Apical | Basal | P$^1$ | Apical | Basal | P$^1$ |
| Leaf      | Adaxial | Abaxial |       | Adaxial | Abaxial |       | Adaxial | Abaxial |       |
| region    |        |        |       |        |        |       |        |        |       |
| Leaf side |         |        |       |         |        |       |         |        |       |
| SR        |         |        |       |         |        |       |         |        |       |
| JA        | 26±1.2$^a$ | 32±1.3$^a$ | 0.026 | 20±1.3$^b$ | 25±1.3$^a$ | 0.009 | 34±1.1$^a$ | 43±1.6$^a$ | 0.406 |
| W         | 34±2.1$^a$ | 39±1.1$^a$ | 0.076 | 24±1$^b$ | 25±1$^b$ | 0.241 | 37±2.8$^a$ | 44±4.1$^a$ | 0.105 |
| AC        | 23±2.1$^b$ | 37±2.3$^b$ | <0.001 | 23±1.4$^a$ | 29±1.4$^a$ | 0.003 | 33±2.2$^a$ | 36±3.2$^a$ | 0.468 |
| P$^2$     | *0.032 | 0.25 |       | 0.058 | 0.172 |       | 0.902 | 0.708 |       |
| T         | 37±1.2$^a$ | 47±3.1$^a$ | 0.002 | 34±1.6$^a$ | 47±2.6$^a$ | <0.001 | 22±1.3$^b$ | 28±2.4$^b$ | 0.044 |
| JA        |         |        |       |         |        |       |         |        |       |
| W         | 51±4.8$^a$ | 57±3.2$^{ab}$ | 0.288 | 40±3.2$^a$ | 51±5.1$^a$ | 0.074 | 32±2.6$^a$ | 66±5.5$^a$ | <0.001 |
| AC        | 37±2.2$^b$ | 59±5.3$^b$ | 0.005 | 34±2$^a$ | 42±2$^a$ | 0.008 | 31±1.8$^a$ | 44±3.3$^a$ | <0.001 |
| P$^2$     | *0.05  | *0.006 |       | 0.474 | 0.596 |       | *0.044 | *0.007 |       |
| Z         | 33±1.7$^a$ | 30±2.3$^a$ | 0.330 | 29±1.4$^b$ | 29±0.5$^b$ | 1.00 | 28±0.4$^a$ | 37±1.1$^b$ | <0.001 |
| JA        |         |        |       |         |        |       |         |        |       |
| W         | 41±2.2$^a$ | 40±2.3$^a$ | 0.673 | 41±3.5$^a$ | 38±1.6$^a$ | 0.693 | 21±1.6$^b$ | 28±1.7$^b$ | <0.001 |
| AC        | 32±1.2$^b$ | 29±1.2$^b$ | 0.175 | 34±3.4$^a$ | 47±4$^a$ | <0.023 | 31±0.5$^a$ | 26±1.4$^a$ | <0.009 |
| P$^2$     | 0.140 | *0.016 |       | *0.01 | *0.036 |       | *0.002 | *0.008 |       |

Note: * indicates significant difference compared to the control (W) at p < 0.05.
Table S3. P values of the comparison of the number of glandular trichomes between apical and basal region of the same leaf, and between nodes in plants grown from seeds from 3 localities: San Rafael (SR), Tecomavaca (T) and Zapotitlán Salinas (Z) treated with 1.5 mL of 1mM jasmonic acid (JA), 1.5 mL of water (W) or 1.5 mL of distilled water + acetone 1% (AC) to the whole plant. Asterics indicate significant differences (Student’s t test <0.05).

|       | Node 1 Young leaves | Node 2 Mature leaves | Apical Region | Basal Region |
|-------|---------------------|----------------------|---------------|--------------|
|       | Apical x Basal      | Apical x Basal       | Node 1 x Node 2 | Node 1 x Node 2 |
|       | Adaxial             | Abaxial              | Adaxial       | Abaxial      |
| SR    | JA                  | *0.007               | *0.015        | 0.146        | 0.789        | 0.274        | *0.015        | *0.008        |
|       | W                   | *<0.001              | *<0.001       | 0.835        | 0.187        | 0.521        | *<0.001       | *<0.001       |
|       | AC                  | 0.895                | *0.006        | 0.246        | 0.796        | *<0.001      | 0.771        | *0.011        | *0.029        |
| T     | JA                  | 0.092                | 0.909         | 0.819        | 0.533        | *<0.001      | *<0.001      | *<0.001       | *<0.001       |
|       | W                   | 0.062                | 0.338         | 0.602        | *<0.001      | *0.002       | *0.001       | *0.046        | 0.087        | 0.070        |
|       | AC                  | 0.252                | *<0.001       | *<0.001      | *0.036       | *0.024       | *0.004       | *<0.001       | *<0.001       | *<0.001       | *0.009        |
| Z     | JA                  | 0.062                | 0.465         | *0.003       | *<0.001      | *0.004       | *0.027       | *0.001        | *0.019        |
|       | W                   | 0.965                | 0.707         | 0.771        | 0.416        | *<0.001      | *<0.001      | *<0.001       | *<0.001       | *0.004        |
|       | AC                  | 0.677                | *<0.001       | *<0.001      | *0.005       | 0.464        | 0.092        | *<0.001       | *0.031        | *0.003        |
Figure S1. Peltate glandular trichomes on the adaxial side of a leaf of *L. graveolens* next to (a) non-glandular trichome (NT) 160X (b) stomata (ST) 500X, and (c) detail of external structure with large unicellular head (H) and short stalk (S) 1600X.
Figure S2. Number of glandular trichomes per mm² on adaxial and abaxial sides from apical and basal region of leaves of *L. graveolens* plants, obtained from seeds from 3 localities: San Rafael, Tecomavaca and Zapotitlán Salinas treated locally with 120 μL 1 mM of jasmonic acid (JA L), 120 μL of distilled water (W) and 120 μL of distilled water + acetone 1% (AC). For a given treatment, bars that share letters are not significantly different (LSD >0.05). Values are means ± SE based on 5 replicates.
Figure S3. Concentration of major terpenes of plants *f L. graveolens*, obtained from seeds from 3 localities: San Rafael (SR), Tecomavaca (T) and Zapotitlán Salinas (Z) treated locally with 120 μL of jasmonic acid 1 mM (Local), 120 μL of distilled water (W), 120 μL of distilled water + acetone 1% (AC), and from adjacent non-treated leaves (Systemic) of a treated plant. Different letters without apostrophe indicate significant differences (LSD ≤0.05), letters with apostrophe indicate differences (LSD ≤0.1). Values are means ± SE based on 5 replicates.
Figure S4. Concentration of major terpenes of plants *L. graveolens*, obtained from seeds from 3 localities: San Rafael (SR), Tecomavaca (T) and Zapotitlán Salinas (Z) sprayed with 1.5 mL of 1 mM jasmonic acid (AJ), 1.5 mL of distilled water (W), or 1.5 mL water + acetone 1% (AC). Samples were taken from first and second basal node (n=15). Different letters without apostrophe indicate significant differences (LSD ≤0.05), letters with apostrophe indicate differences (LSD ≤0.1). Values are means ± SE based on 5 replicates.
Figure S5. Principal component analysis (PCA) ordination diagram with leaf samples from *L. graveolens*, obtained from seeds from 3 localities: San Rafael (SR –––), Tecomavaca (T–––) and Zapotitlán Salinas (Z–––) and the concentration of terpenes (Ter1:o-cymene, Ter2:Thymol, Ter3:carvacrol, Ter4:caryophyllene), density of glandular trichomes on adaxial side in the apical part of the leaf (TRADAP), adaxial side in the apical basal of the leaf (TRADBA) and abaxial side in the apical part of the leaf (TRABAP). The fraction of the total variance accounted for by each axis is indicated. n=90.
References

Abu-Lafi S, Odeh I, Dewik, H, Qabajah M, Hanus LO, Dembitsky VM. 2008. Thymol and carvacrol production from lives of wild palestinian *Majorana syriaca*. Bioreour Technol. 99:3914-3918.

Babst BA, Sjödin A, Jansson S, Orians CM. 2009. Local and systemic transcriptome responses to herbivory and jasmonic acid in *Populus*. Tree Genet Genomes. 5:459-474.

Canales M, Hernández T, Caballeto J, Romo de Vivar A, Avila G, Duran A, Lira R. 2005. Informant consensus factor and antibacterial activity of the medicinal plants used by people of San Rafael Coxcatlán, Puebla, México. J Ethnopharm. 97:429-439.

Casas A, Valiente-Banuet A, Viveros JL, Caballero J, CorTeas L, Dávila P, Lira R, Rodríguez I. 2001. Plant resources of the Tehuacán-Cuicatlán Valley, Mexico. Econ Bot. 55:129-166.

Grupo Mesófilo. 2001. Ordenamiento territorial de la comunidad de Santa María Tecomavaca, Teotitlán, Oaxaca. Etapa 1: Diagnóstico y sistematización de la información de la comunidad [Land-use plan in Santa María Tecomavaca community, Teotitlán, Oaxaca. Stage 1: Diagnosis and systematization of community information]. [accessed 2018 November 19][28 p.]. Spanish. http://www.grupomesofilo.org/pdf/proyectos/OTC/OTC_Tecomavaca.pdf

Hernández T, Canales M, Avila JG, Durán A, Caballero J, Romo de Vivar A, Lira R. 2003. Ethnobotany and antibacterial activity of some plants used in traditional medicine of Zapotitlán de las Salinas, Puebla, Mexico. J Ethnopharm. 88:181-188.

López-Galindo F, Muñoz-Iniestra D, Hernández-Moreno M, Soler-Aburto A, Castillo-López MC, Hernández-Arzate I. 2003. Análisis integral de la toposecuencia y su influencia en la distribución de la vegetación y la degradación del suelo en la subcuenca de Zapotitlán Salinas, Puebla [Comprehensive analysis of the toposequence and its influence on the distribution of vegetation and soil degradation in the sub-basin of Zapotitlán Salinas, Puebla]. Bol Soc Geol Mex 56:19-41. Spanish

Osorio-Beristain O, Valiente-Banuet A, Dávila P, Medina R. 1996. Tipos de vegetación y diversidad β en el Valle de Zapotitlán de las Salinas, Puebla, México [Types of vegetation and β diversity in the Zapotitlán de las Salinas Valley, Puebla, México]. Bol Soc Bot Mex. 59:35-58. Spanish.