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Short Communication

Physiological response of *Lactuca sativa* exposed to 2-nonanone emitted by *Bacillus* sp. BCT9

Paola Fincheira[a,b], Andrés Quiroz[b,c,*]

[a] Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Temuco, Chile
[b] Centro de Excelencia en Investigación Biotecnológica Aplicada al Medio Ambiente (CIBAMA), Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco, Chile
[c] Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco, Chile

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ABSTRACT

Volatile organic compounds (VOCs) released from bacterial species have been reported as plant growth inducers. In this sense, *Lactuca sativa* was used as model vegetable to prospect the effects of 2-nonanone released by *Bacillus* sp. BCT9 at cellular and organ structure level, so we present preliminary results about the physiological effects. In this study, 2-day-old *L. sativa* were exposed to 2-nonanone for 10 days under two delivery systems: 1) 2-nonanone (abrupt delivery) and 2) 2-nonanone + lanolin (controlled delivery). The X-ray elemental microanalysis, scanning electron and confocal laser microscopies techniques were used to evaluate physiological changes "in vivo" conditions. The results indicated that 2-nonanone increased root and shoot length independently of 2-nonanone delivery system after 7 days of exposition. Additionally, 2-nonanone elicited the increase of anthocyanin and not affects chlorophyll content and electrolyte leakage percentage. The abrupt delivery elicited the increase of both length and density of root hair without causing changes in size of cell epidermis, while controlled delivery induced stomatal opening. Besides, 2-nonanone exposition did not modify the composition and distribution of carbon, nitrogen, phosphorus, potassium, and chlorine in the surface of plant tissue. The results suggested that 2-nonanone acts as a bacterial signal molecule to elicit changes related to root development without damaging the external morphology while epidermal cells at leaf level are not affected, suggesting that 2-nonanone can be an important tool to apply to vegetables.

1. Introduction

Volatile organic compounds (VOCs) emitted by bacterial species have been reported as a signal molecule that induce plant growth modulating essential nutrients, hormonal balance, metabolism and sugar concentration of plants (Kanchiswamy et al., 2015). VOCs are characterized by having low molecular weight (< 300 g/mol), high vapor pressure (10 hPa at 20 °C), low boiling point and belonging to diverse chemical classes (alcohols, acids, ketones, terpenes, hydrocarbons, among others) (Audrain et al., 2015). Currently, there is important evidence supporting the role of bacterial VOCs in plant growth induction (Kanchiswamy et al., 2015). The study performed by Ryu et al. (2003) showed that 2,3-butanediol released by *Bacillus subtilis* GB03 induced the increase of surface leaf area in *Arabidopsis thaliana*. Furthermore, Velázquez-Becerra et al. (2011) revealed that dimethylhexadecylamine emitted by *Arthrobacter agilis* UMCV2 increased fresh weight, stem length, root length and lateral root density on *Medicago sativa*. Additionally, exhaustive studies performed on a model interaction constituted by *B. subtilis* GB03 and *A. thaliana* have shown that VOCs induced relevant changes at metabolism level in plant. The study carried out by Zhang et al. (2007) indicated that genes related to metabolism, growth, stress, hormone regulation and cellular signaling are modulated by the mixture of GB03 volatiles. Furthermore, a study developed by Kwon et al. (2010) showed that chlorophyll content and proteins expressions at subcelullar level (i.e. cytosol, cell wall and mitochondrion), as well as molecular functions (i.e. transport, antioxidant, oxidoreductase) and biological processes (i.e. responds to stimulus, photosynthesis and nitrogen metabolism) were regulated after VOCs emitted by GB03.

These studies suggested that VOCs emitted by bacterial species can be used to induce growth in horticultural species as a novel alternative. Nevertheless, there is scarce information associating morphological and physiological effects with a specific VOC in vegetables. Hence, *Lactuca sativa* emerges as an interesting model vegetable to evaluate bioactive

* Corresponding author at: Avenida Francisco Salazar #01145, Laboratorio de Química Ecológica, Universidad de La Frontera, Temuco, Chile.
E-mail address: andres.quiroz@ufrontera.cl (A. Quiroz).

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VOCs because it is considered as inexpensive, simple, reliable and sensitive specie to evaluate phytotoxicity (Charles et al., 2011). Previously, a study performed by Fincheira et al. (2016) reported that VOCs emitted by *Bacillus* sp. BCT9 increased the growth on *L. sativa* at radical and shoot level. Afterwards, Fincheira et al. (2017) showed that 2-nonanone is a bacterial bioactive compound eliciting the highest growth-inducing activity on seedlings. The prospection of action mechanisms triggered by 2-nonanone on *L. sativa* has not been elucidated so far. Therefore, this study was focused in the evaluation of agronomic parameters over time and in the microscopic prospection of radical and foliar tissues through techniques that allow "*in vivo*" analysis to elucidate the possible target pathways activated by 2-nonanone for inducing *L. sativa* growth.

2. Materials and methods

2.1. Plant material and growth conditions

Commercial seeds of *L. sativa* (Green lettuce cv Reina de mayo asepo, semillas Fito S.A) were sterilized for 8 min with 3% sodium hypochlorite and washed with sterile distilled water. Later, seeds were germinated on Murashige and Skoog basal medium with vitamins 0.5X (PhytoTechnology Laboratories, LLC™) containing 0.8% agar and 1.5% sucrose (MS-A). Petri dishes were placed under controlled conditions (16/8-h light-dark cycle, T°20–25 °C). Seedlings were transferred into two-compartment Petri dish after 2 days for experimental bioassays (Minerdi et al., 2011).

2.2. L. sativa growth induced by 2-nonanone

Two 2-day-old *L. sativa* seedlings were placed into a two-compartment Petri dish (90 × 15 mm) containing MS-A. In the opposite compartment containing the same medium was placed a sterile paper disk with 2-nonanone. Two treatments were employed to apply volatile: 1) 50 ppm of 2-nonanone diluted in hexane (abrupt delivery) and 2) 50 ppm of 2-nonanone diluted in hexane + lanolin solution (0.16 g mL^{-1}) (ratio 1:1) (controlled delivery). Non-exposed seedlings to 2-nonanone were used as a control. Seedlings were exposed to 2-nonanone for 10 days (Zou et al., 2010).

2.3. Contents of total chlorophyll and anthocyanin

To measure total chlorophyll content on *L. sativa*, 500 mg of leaf samples were extracted with 5 mL 80% (v/v) aqueous acetone in dark conditions, and the respective absorbance of the extracted chlorophyll was recorded at wavelengths of 645 and 663 nm in spectrophotometer UV/Vis. The chlorophyll (Chl) concentrations were determined using the protocol described by Lichtenthaler and Wellburn (1983), using the following equations: Chl A (mg/g): (12.72 A663–2.59A645) × V/ (W × 1000) and Chl B (mg/g): (22.88 A663–4.67A665) × V/ (W × 1000), where V is the volume constituted by acetone extract (mL) and W is fresh weight of leaf sample (mg). The total anthocyanin content was determined according to pH differential method. The vegetable tissue (0.05 g) was placed in 1 mL of methanol containing 1% HCl. Later, the samples were shaken at 4 °C overnight. Then, samples were diluted in buffers pH 1 (25 mM KCl) and pH 4.5 (0.4 M CH_3COONa). The absorbances of buffers pH 1 and pH 4.5 with diluted samples were measured at 510 nm and 700 nm, respectively. The anthocyanin (Ant) content was calculated as follows: Ant (mg/g): 10 × M × F/ K × $[(A510 - A700)_{pH\ 1} - (A510 - A700)_{pH\ 4.5}]$ (Steidle Neto et al., 2016).

2.4. Analyses of electrolyte leakage

The electrolyte leakage percentage (EL%) was measurement according to the method reported by Omezzine et al. (2014) with minor modifications. The leaves were placed in 10 mL of deionized water and incubated in agitation at room temperature for 24 h. Initial electrical conductance (C_1) was determined using a PCE-PHD 1 conductivity meter. Then, samples were autoclaved at 120 °C for 20 min and the final electrical conductivity (C_2) was obtained after cooling at 20 °C. The EL was calculated according to the following equation: EL (%) = $C_1/C_2 \times 100$.

2.5. Analysis of L. sativa morphology

Analyses of tissues were performed through scanning electron microscopy (VP-SEM SU 3500 Hitachi-Japan). Images were captured with backscattered electron (BSE) detector and working distance (WD) of 10.5 mm for leaf or 5 mm for root, the beam energies used were 10.5 KeV (leaf) or 5 KeV (root) to observe samples at 6 Pa (Angeles et al., 2004). The ImageJ software was used to determine the root hair length, size of root epidermal cells and length of stomatal aperture through the reference scale provided by SEM photograph. In addition, root density was determined through the number of root hairs for 100 µm of primary root observed in the photograph. The open and closed stomata were determined in the same leaf surface study of 420 × 298 μm^2, according to the treatment applied.

Scanning electron microscopy coupled to X-ray elemental microanalysis with energy-dispersive X-ray spectroscopy (EDS) detector was used to evaluate distribution and intensity of elements present in the surface of tissues. Leaf tissue analyzes were performed by Confocal Laser Microscopy (CLSM FV 1000 Olympus-Japan), where the samples were incubated for 30 min with 0.1% w/v safranin at 4 °C under darkness conditions and washed with sterile distilled water to visualize cell wall through λ excitation/emission 546/590 nm and autofluorescence of chlorophyll through 488/530 nm and 633 / 650–750 nm (Kodama, 2016). Furthermore, an exhaustive study about the stomatal opening was observed "*in vivo*" through scanning electron microscopy and Confocal Laser Microscopies (different fields of observation were photographed on the leaf surface) immediately after the bioassays was completed.

2.6. Image and statistical analysis

The ImageJ software was used to perform plant growth measurements. The results obtained after treatment applications were analyzed by Statistix v10 using analysis of variance (ANOVA) and LSD test ($P \leq 0.05$).

3. Results

3.1. The growth induction on L. sativa elicited by 2-nonanone

The results showed that 2-nonanone emitted by *Bacillus* sp. BCT9 has a relevant role to induce *L. sativa* growth, showing relevant effects during the first 10 days of exposition. The Fig. 1a shows that shoot length was increased by 2-nonanone with abrupt delivery on day-5, while both applications (abrupt and controlled) increased this parameter on day-10 (~9-20%). Besides, root length was increased ~18% after day-7 and 10 through both applications of the volatile (Fig. 1b). The Fig. 1c shows that fresh weight did not increase after exposure to 2-nonanone, reaching 0.025 g approximately. The Fig. 1d shows that seedlings exposed to 2-nonanone did not show stress signs, which was measurement through EL%, where the values reach about ~250 µS in all treatments. Furthermore, the anthocyanins content was strongly increased after exposition to 2-nonanone with abrupt delivery, reaching 0.89 and 0.67 mg/g during abrupt and controlled release, respectively (Fig. 1e). Nevertheless, chlorophyll content was not regulated after exposure to 2-nonanone, reaching around 0.03 mg/g (Fig. 1f).

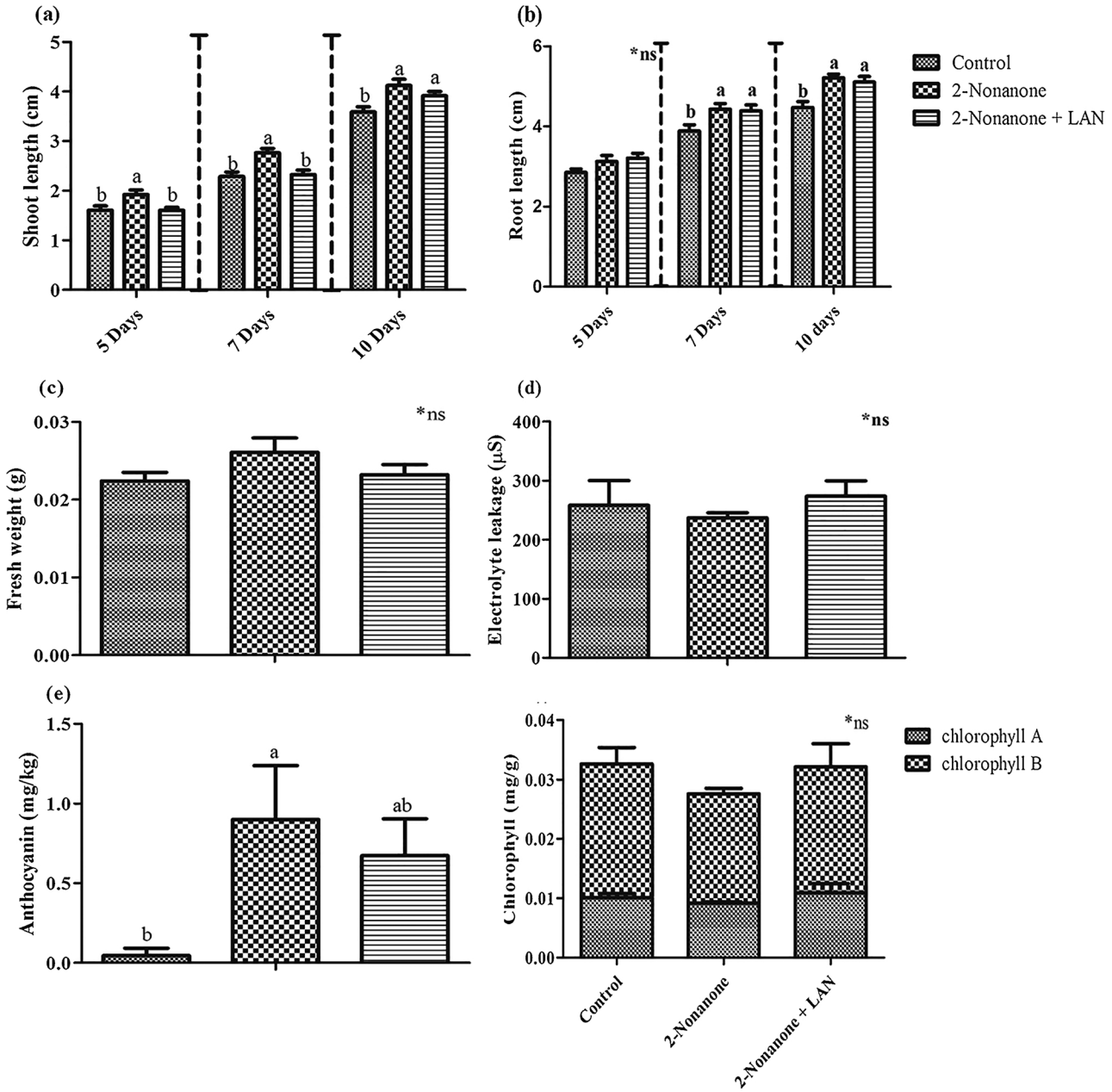

Fig. 1. Effects of 2-nonanone on *L. sativa* growth. Seedlings were placed on Murashige & Skoog medium and exposed to two delivery volatile systems: 2-nonanone (abrupt) and 2-nonanone + lanolin (controlled), both with 50 ppm of bioactive compound. Plant growth parameters evaluated were (a) shoot length, (b) root length, (c) fresh weight, (d) electrolite leakage percentage (%), (e) anthocyanin and (f) chlorophyll. Error bars indicate error standard of mean (N = 20). The letters indicate differences statistically significant according to T-Student test ($p < 0.05$) performed for each delivery system (*ns = no significant difference).

3.2. Morphological analysis of root and shoot of L. sativa after exposition to 2-nonanone

The results indicated that 2-nonanone increased the root hair development (Fig. 2a–c). Seedlings exposed to abrupt release of 2-nonanone elicited 106.8% increase in root hair density (Fig. 2d). Root hair length increased 105.5% in seedlings exposed to the same treatment respect to control, reaching 421.7 µm respectively (Fig. 2e) and no changes were observed in the size of radical epidermal cells (Fig. 2f). At foliar level, the results indicated that controlled delivery of 2-nonanone had relevant effects to induce stomata aperture (Fig. 3a–f). The *L. sativa* seedlings increased the number of open stomata (leaf surface field examined: 420 × 298 µm), presenting 384% per cm^2 of increasing respect to control (Fig. 3g). Moreover, the length of stomata aperture tended to increase after the application of 2-nonanone with controlled delivery, but no statistically significant difference was found (Fig. 3h). Additionally, an approach of chlorophyll fluorescence was used to visualize morphological chloroplast state indicating that they were not altered under 2-nonanone exposition.

To complement the information provided by microscopic analysis regarding the surface morphology, a scanning electron microscopy coupled to X-ray elemental microanalysis was carried out (Fig. 4a–f). The results indicated that 2-nonanone exposition did not alter the distribution and composition of carbon, nitrogen and micro elements in the surface of root and leaf, independently of delivery system applied (Fig. 4g, h). Nevertheless, the oxygen is slightly modified after exposition to 2-nonanone with controlled delivery in the surface of root. The N and P are found in the same proportion in all treatments respect to control at both levels, indicating that 2-nonanone did not modify normal physiological processes associated to the assimilation of

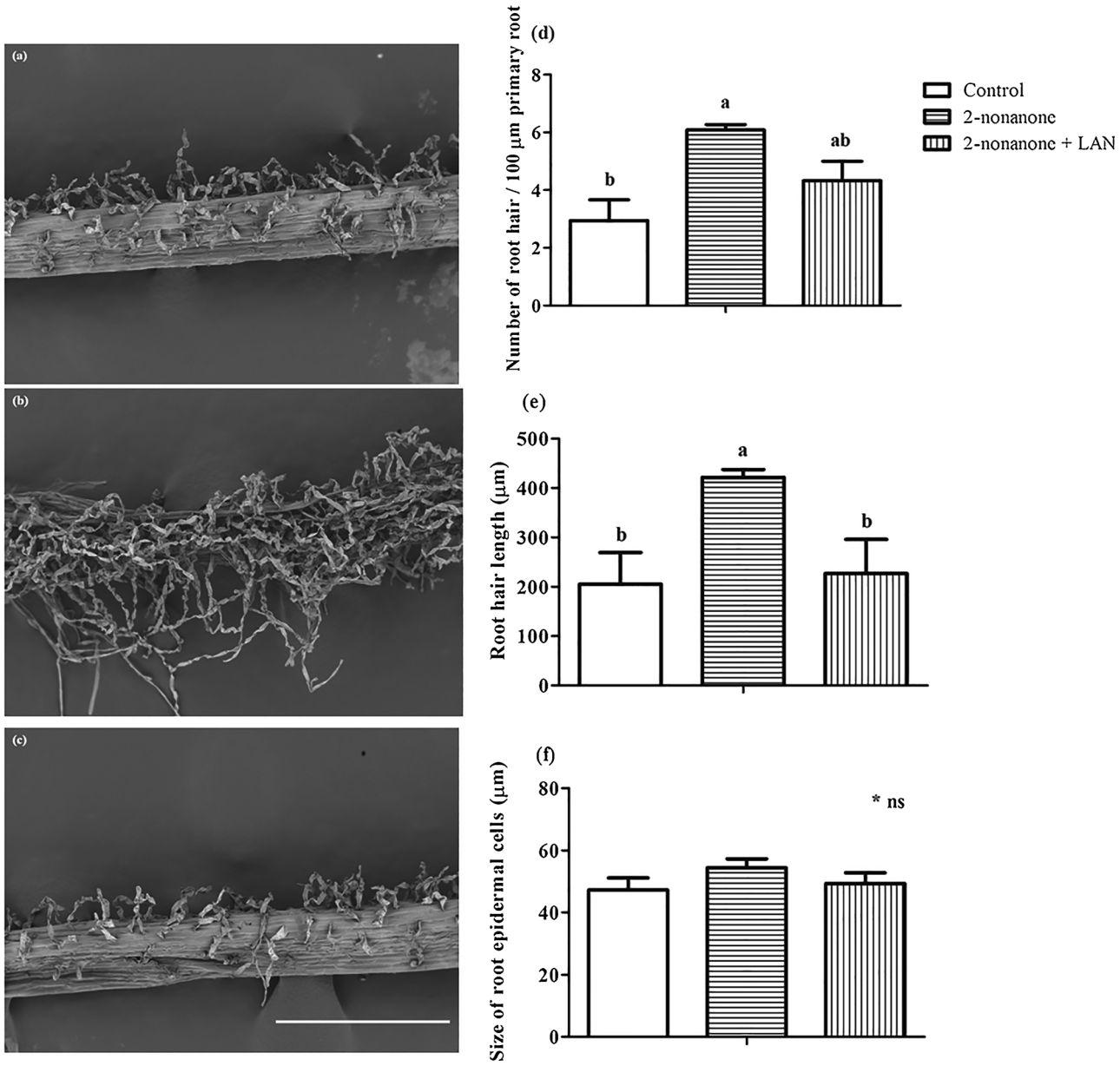

Fig. 2. Root hair development on *L. sativa* after 10 days of exposure to 2-nonanone. The *L. sativa* seedlings were placed in the surface of Murashige & Skoog medium. (a) Control, (b) nonanone (abrupt system), and (c) 2-nonanone + lanolin (controlled system). Parameters evaluated were (d) root hair density, (e) root hair length and (f) size of root epidermal cells. Error bars indicate error standard of mean (N = 3). The letters indicate differences statistically significant according to ANOVA (LSD test, $p < 0.05$). Scalebars: 400 µm.

nutrients. Furthermore, Fig. 4i showed the elements related to stomatal opening, where Ca^{+2} was strongly increased in the surface of tissue after exposition to controlled delivery of 2-nonanone.

4. Discussion

VOCs are carbon-containing compounds with physico-chemical properties that allow their emission to the atmosphere making them ideal molecules to elicit physiological responses on plant target (Dudareva et al., 2013; Kanchiswamy et al., 2015). The first evidence about the relevant effects of bacterial volatiles on plant growth induction was reported by Ryu et al. (2003), who showed that 2,3-butanediol released by *B. subtilis* GB03 elicited the growth of surface leaf of *A. thaliana* by activation of cytokinin signaling. Later, Bailly et al. (2014) showed that indole release by *Escherichia coli* modulates the secondary root development of the same plant species through auxin activity. In addition, Castulo-Rubio et al. (2015) reported that volatiles emitted by *A. agilis* UMCV2 induce the increase of Fe acquisition in *Sorghum bicolor* through the induction of iron reduction (Fe^{+3} to Fe^{+2}) and transport of Fe^{+2} into the cell plant.

Until now, molecular biology techniques have been intensely used for understanding the possible mechanisms associated to growth inducing activity of bacterial volatiles (Zhang et al., 2007; Kwon et al., 2010). Under this context, the study was focused on the prospection of physiological effects induced by 2-nonanone on *L. sativa* seedlings. The data indicated that 2-nonanone emitted by *Bacillus* sp. BCT9 has a relevant ecological role to induce growth on *L. sativa* at foliar and root level, simultaneously during the first 10 days of exposition.

The results related to growth induction through time by bacterial volatile indicated that the effect was found on day-7 at shoot and root level, in concordance with the report from Orozco-Mosqueda et al. (2013) and Vaishnav et al. (2015) in *Medicago truncatula* and *Glycine*

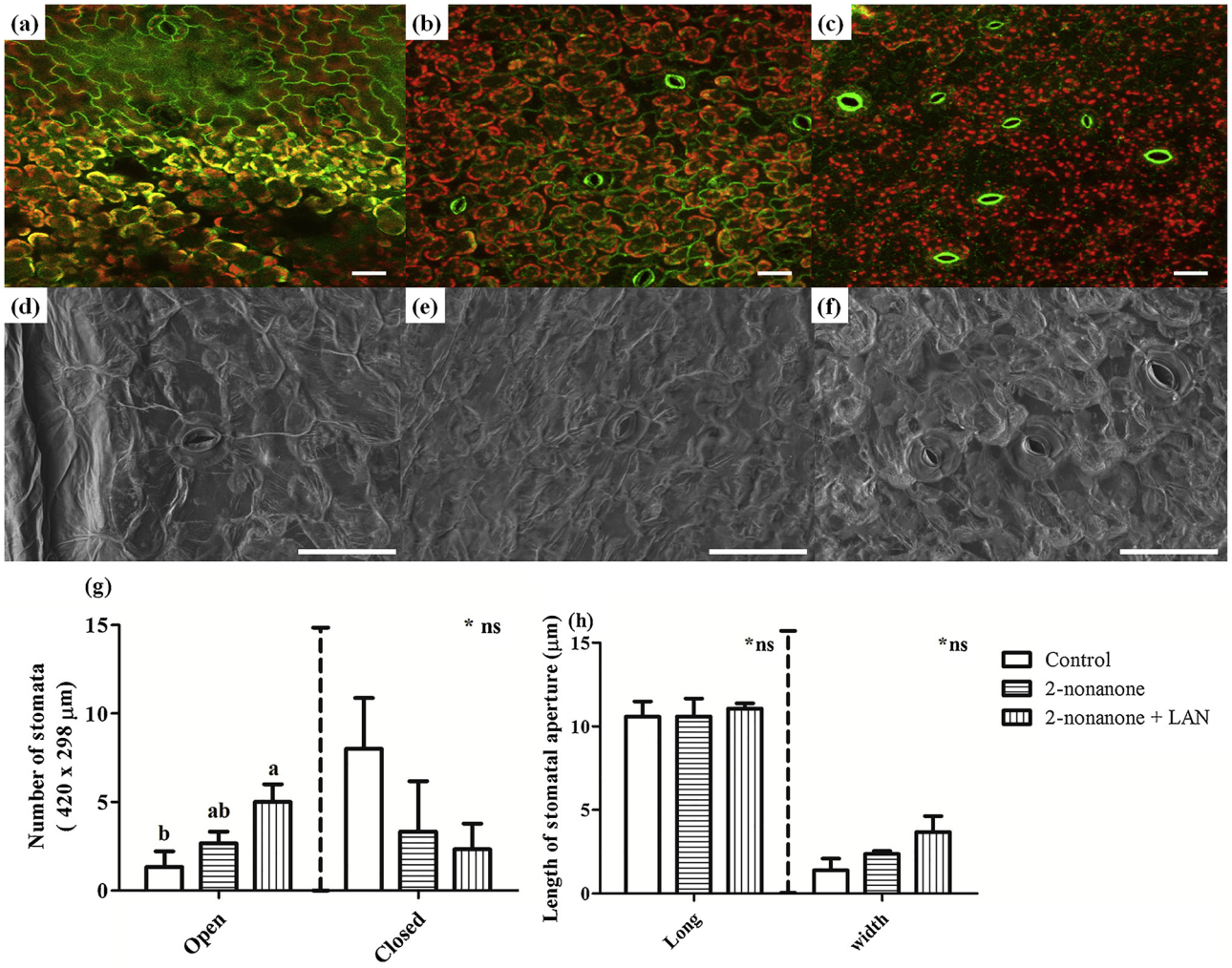

Fig. 3. Photographs of leaf surface of *L. sativa* seedlings exposed to 2-nonanone during 10 days captured through Confocal and Scanning Electron microscopies. The *L. sativa* seedlings were placed in the surface of Murashige & Skoog medium: (a, d) control, (b, e) 2-nonanone (abrupt system) (c, f) and 2-nonanone + lanolin (controlled system). Parameters evaluated were (g) Number of open and closed stomata (field surface: 420 × 298 µm) and (h) length of stomatal aperture. Error bars indicate error standard of mean (N = 3). The letters indicate differences statistically significant according to ANOVA (LSD test, $p < 0.05$) (*ns = no significant difference).

max. The EL% is an important physiological parameters reflexing the stress response of plant cells and it is highly used as test for stress-induced injury (Assaha et al., 2016). The EL is measured in different species, tissues, and cell types, and it can be triggered by diverse stress factors, including pathogen attack, salinity, heavy metals, and oxidative stress, among others. The data indicated that 2-nonanone does not induce a stress perceptible through EL, which would indicate preliminary that this bacterial VOC does not alter the seedling physiologically. Furthermore, photosynthetic pigments were analyzed to investigate the role of bacterial to volatiles in the photosynthesis process. The results indicated that the concentration of anthocyanins were increased after the exposition to abrupt delivery of 2-nonanone, which indicates it important role to trigger cellular signals that induce their increase. In addition, it is noteworthy that anthocyanins are water soluble pigments with important role to protect chloroplast from damage induced by photo-oxidative agents as reactive oxygen species, for which they are essential for the plant survival under stress conditions (Silva et al., 2016). In contrast, the chlorophyll content was not affected after exposition to 2-nonanone on day-10; but others studies indicated the relevant role of bacterial volatiles to increase the chlorophyll concentration (Zhang et al., 2009; Orozco-Mosqueda et al., 2013).

The prospection of physiological effects induced by 2-nonanone on *L. sativa* seedlings using microscopic techniques were used to obtain the preliminary results related to changes in cell or organ structures at root and foliar level. The root system plays an important role in plant support and acquisition of water and minerals (Ruiz-Herrera et al., 2015). The results suggest an ecological role of 2-nonanone to increase the radical absorption zone by enhancing the density and length of root hair of *L. sativa* seedlings. Therefore, bacterial volatiles could provide the best conditions to soil environmental adaptation and exploratory capacity, which can be associated to the activation of pathways associated to root development i.e. phytohormones and nutrients availability (Ruiz-Herrera et al., 2015). However, further studies are required to elucidate the specific action pathway activated on *L. sativa* after exposition to 2-nonanone. The leaf is the organ associated to photosynthesis process, where the plant captures the light energy to produce glucose. In the leaf surfaces are found the stomata, which are a small specialized cells formed by two epidermal guard cells localized on the leaf surface with importance in the regulation of both gases O_2 and CO_2 flow, and water loss (Kollist et al., 2014).The results indicated the importance of delivery system to induce the stomata aperture, where controlled conditions for the release is essential to increase the number of stomata aperture, by which 2-nonanone and others bacterial volatiles can play an important role in the flow of gases into and out of cells.

To complement the information provided by microscopy analysis regarding the surface morphology, a scanning electron microscopy

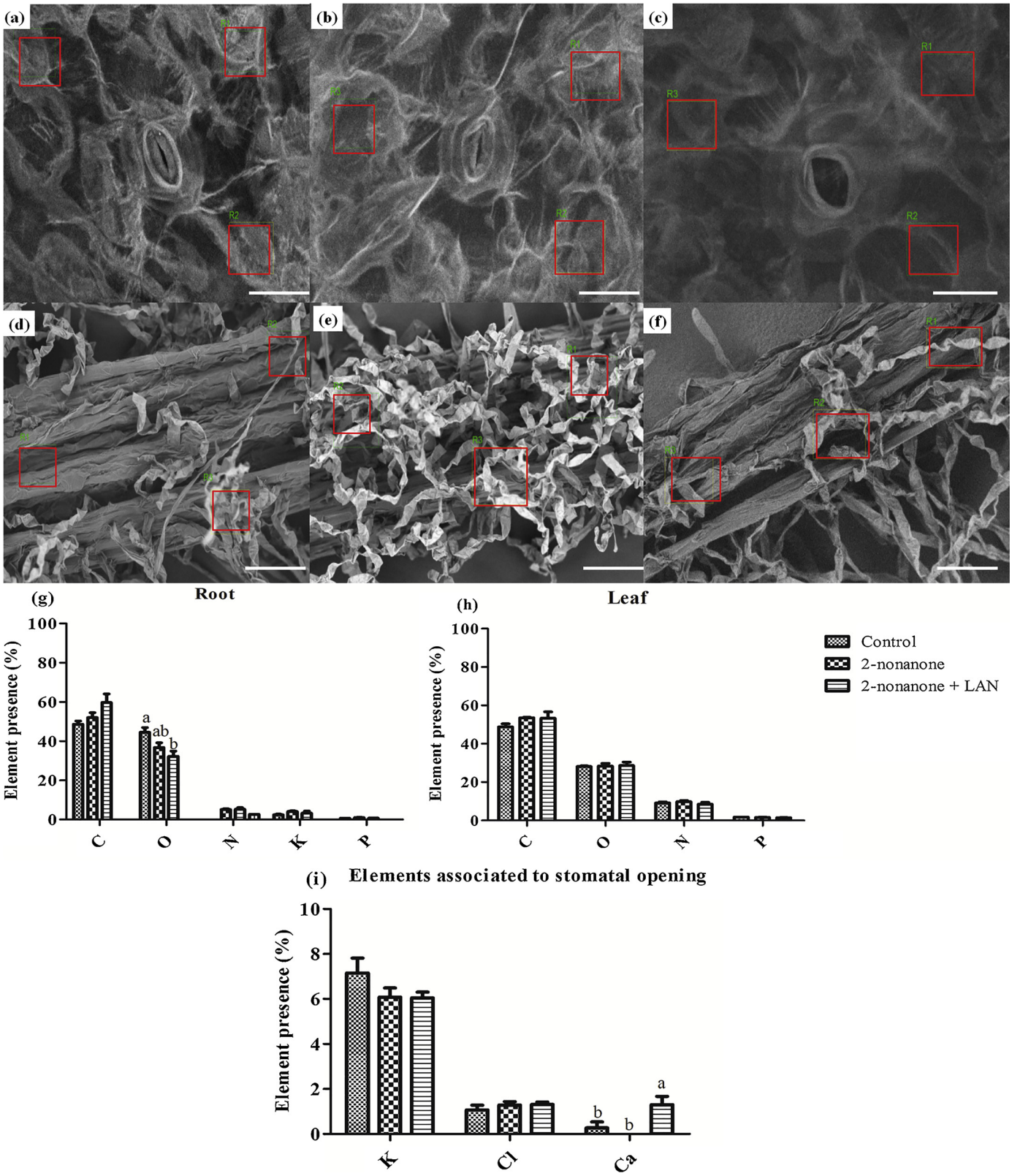

Fig. 4. Percentage of elements presents on the surface of *L. sativa* seedlings at root and leaf level analyzed by Scanning electron microscopy coupled to X-ray elemental microanalysis after 10 days of exposition to 2-nonanone. Red squares are representative zones of surface of leaf and root tissues exposed to (a, d) control, (b, e) 2-nonanone (abrupt), and (c, f) 2-nonanone + lanolin (controlled). Surface elements associated to (g) root, (h) leaf and (i) stomatal opening were evaluated. Mean ± standard deviation (N = 3). (*ns = no significant difference).

coupled to X-ray elemental microanalysis was carried out to perform microcaracterization of elements present in the surface of *L. sativa* seedlings in three zones distributed in a specific micro-zone. The data showed that the percentage of elements presence was not altered after volatile exposition at root and leaf level with the exception of oxygen (root). The percentage of C and O are characterized by being provided by water and assimilated biochemically through carboxylation and oxide-reduction reactions, while the macronutrients (NPK) are acquired from culture substrate. It is emphasized that Ca^{+2} was strongly increased in the surface of leaf tissue after exposition to controlled delivery of 2-nonanone, suggesting the induction of Ca expulsion from cytosol to the cell outside to promote the stomata opening (Kollist et al., 2014).

In summary, the results showed the active role of 2-nonanone with abrupt delivery to induce growth on *L. sativa* seedlings by increasing the absorption surface of nutrients through the induction of length and root hair density. Furthermore, the controlled delivery induced stomata aperture, suggesting that 2-nonanone can interact with this structure, in accordance with the model described by Widhalm et al. (2015). Furthermore, Matsui (2016) reported that VOCs from the atmosphere entered intra cellular space (mesophyll) through stomata, indicating that VOCs are partitioned between gas and liquid phase inside the cell, depending on the physicochemical characteristics of the compound involved and metabolized in the cytosol. To our knowledge, this is the first study that provides experimental evidence about the significant physiological effects of volatile organic compound emitted by *Bacillus* species, such as 2-nonanone on *L. sativa* seedlings through visual tools. According to these results and the bibliography, we propose the possible action role of a VOC on *L. sativa* seedlings, suggesting that the controlled delivery of 2-nonanone elicited the stomata aperture improving the gas exchange and the photosynthetic process; or this compound could be incorporated into the plant and subsequently transported to the radical zone to exert their action on growth promoter.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.11.002.

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