Volume 16, Issue 1, January 2023
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Hellenic Plant Protection Journal

A semiannual scientific publication of the
BENAKI PHYTOPATHOLOGICAL INSTITUTE

Editorial Policy

The *Hellenic Plant Protection Journal (*HPPJ) (ISSN 1791-3691) is the scientific publication of the Benaki Phytopathological Institute (BPI) replacing the *Annals of the Benaki Phytopathological Institute* (ISSN 1790-1480) which had been published since 1935. Starting from January 2008, the *Hellenic Plant Protection Journal* is published semiannually, in January and July each year.

HPPJ publishes scientific work on all aspects of plant health and plant protection referring to plant pathogens, pests, weeds, pesticides and relevant environmental and safety issues. In addition, the topics of the journal extend to aspects related to pests of public health in agricultural and urban areas.

Papers submitted for publication can be either in the form of a complete research article or in the form of a sufficiently documented short communication (including new records). Only original articles which have not been published or submitted for publication elsewhere are considered for publication in the journal. Review articles in related topics, either submitted or invited by the Editorial Board, are also published, normally one article per issue. Upon publication all articles are copyrighted by the BPI.

Manuscripts should be prepared according to instructions available to authors and submitted in electronic form on line at http://www.hppj.gr. All submitted manuscripts are considered and published after successful completion of a review procedure by two competent referees.

The content of the articles published in HPPJ reflects the view and the official position of the authors. The information and opinions contained herein have not been adopted or approved by the HPPJ Editorial Board. The HPPJ Editorial Board neither guarantees the accuracy of the information included in the published articles nor may be held responsible for the use to which information contained herein may be put.

For all parties involved in the act of publishing (the author(s), the journal editor(s), the peer reviewers, and the publisher) it is necessary to agree upon standards of expected ethical behavior. HPPJ follows the ethics statements of De Gruyter journals, which are based on the Committee on Publication Ethics (COPE) Code of Conduct guidelines available at www.publicationethics.org.

For back issues, exchange agreements and other publications of the Institute contact the Library, Benaki Phytopathological Institute, 8 St. Delta Str., GR-145 61 Kifissia, Attica, Greece, e-mail: m.kitsiou@bpi.gr.

This Journal is indexed by: *AGRICOLA, CAB Abstracts*-Plant Protection Database, *INIST* (Institute for Scientific and Technical Information) and *SCOPUS*.

The olive tree of Plato in Athens is the emblem of the Benaki Phytopathological Institute

sciendo

Hellenic Plant Protection Journal **16:** 1-11, 2023
DOI 10.2478/hppj-2023-0001

Evaluation of *in-vitro* antifungal activity against *Fusarium incarnatum* of binary and ternary combinations of lemongrass, garlic and mustard oil-encapsulated lipid nanoemulsions

Minh-Hiep Nguyen* and Thi-Ngoc-Mai Tran

Summary Encapsulated lipid nanoemulsions (EO-LNs) from garlic oil, lemongrass oil and mustard oil were prepared by a combined method of homogenization and sonication with the aim to generate highly effective formulations against *Fusarium incarnatum* (laboratory bioassays). Their combined binary and ternary formulations (preparations by mixing an equal volume ratio of each EO-LNs) were also tested. The synergistic/additive/antagonistic antifungal effect of the EOs (under nanoform) in their combined formulations was determined using the SynergyFinder software with the Bliss independence model. Results revealed the synergistic effect of the combined binary and ternary formulations of garlic oil-encapsulated LNs (NaG), lemongrass oil-encapsulated LNs (NaL) and mustard oil-encapsulated LNs (NaM). Furthermore, the ternary combination, at the same concentration of each constituent EO, had higher antifungal activity than the binary combinations. Nonetheless, at 600 times dilution the NaLG (binary combination) inhibited 96% the mycelial growth of *F. incarnatum*, which was significantly higher than the efficacy of NaMLG (ternary combination) in the same dilution. This could be possibly attributed to the 1.5-time higher concentration of each constituent EO in the binary combination compared to that in the ternary formulation. In addition, NaLG, even at the high EO concentration of 0.4 g/L, did not show any phytotoxicity symptoms on lettuce plants.

Additional keywords: Antifungal activity, essential oils, synergistic effect

Introduction

Every year, pathogenic microorganisms cause a large loss of crop yields (Jiang *et al.,* 2020). *Fusarium incarnatum* is an important fungal pathogen that causes diseases of the root, stem, leaves, and seed, leading to the reduction of the quality and quantity of the yield of many crops such as rice, banana, sorghum, and maize (Akram *et al.,* 2019). Nowadays, chemical fungicides are commonly used to control this fungal pathogen (Yang *et al.,* 2019). Despite their benefits, their use has been linked to environmental pollution, toxicity issues to humans and other organisms, and resistance development of *Fusarium* strains, thus making it difficult to control the pathogen (Zubrod *et al.,* 2019). Therefore, the use of biological methods to control plant diseases is an urging alternative solution.

Plant essential oils (EOs) are increasingly used to control plant diseases caused by fungi (Omar *et al.,* 2019). This is because EOs are eco-friendly, biodegradable with negligible or no toxicity to mammals and plants, and highly effective against a wide range of fungal diseases (Omar *et al.,* 2019; Amini *et al.,* 2012). Furthermore, EOs, as other biofungicides, have a multiple mechanism of action, hence they are less prone to the development of resistance of pathogenic fungi (Gressel *et al.*, 2020). Lemongrass oil with geranial (42.2%) and neral (31.5%), garlic oil with diallyl disulfide (27.1–46.8%) and diallyl trisulfide (19.9–34.1%), and mustard oil with allyl isothiocyanate (71.06%), cyclopropyl isothiocyanate (12.16%) and furfural (3.36%) have been previously demonstrated high fungicidal activity (Satyal *et al.*, 2017; Boukhatem *et al.,* 2014; Peng *et al.,* 2014). Other studies showed that isothyocynate and gerani-

Center of Radiation Technology and Biotechnology, Nuclear Research Institute, 01 Nguyen Tu Luc Street, Dalat city, Lam-dong province, Vietnam.
* Corresponding author: jackminhhiep@yahoo.com

al could strongly inhibit the growth of fungi such as *Fusarium graminearum, Fusarium verticillioides, Fusarium sambucinum, Alternaria alternata* and *Verticilium dahliae* (Drakopoulos *et al.,* 2020; Roselló *et al.,* 2015). Diallyl disulfide and diallyl trisulfide were found to possess a strong fungicidal activity for a broad spectrum of fungi (Wang *et al.,* 2019). Kamsu *et al.* (2019) reported that lemongrass oil inhibited the mycelial growth and conidia germination of *F. incarnatum* at a high concentration of 500 µL/L and 185 µL/L, respectively. Although the antifungal activity of garlic oil and mustard oil against *F. incarnatum* is still unknown, their effects on other *Fusarium* species at high concentrations have been reported (Dutta *et al*., 2021; Wang *et al.*, 2020; Hayat et al., 2016).

High antifungal activities of EOs may be due to their ability to destroy the cellular structure of hyphae and cell organelles, leading to the leakage of both cytoplasm and macromolecules. Some EOs can cross the cell membrane, interacting with the enzymes and proteins of the membrane, thereby producing a flux of protons towards the cell exterior which induce changes in the cells and, ultimately, cell death (Omidbeygi *et al.,* 2007). Silva *et al.* (2008) indicated the inhibitory effect of lemongrass oil against *Candida* spp., because the oil is able to form a charge-transfer complex with an electron donor of fungal cells, resulting in fungal death.

Neverhteless, the practical application of EOs in fungal control is challenging as EOs seem not to exhibit a strong antifungal activity at low concentrations due to low water solubility and poor penetration into plants, they are easily degradable under adverse environmental conditions and their price is often high (Nguyen *et al*., 2020; Kamsu *et al*., 2019; Rao *et al.,* 2005). To overcome these drawbacks, essential oil-encapsulated lipid nanoemulsions (EO-LNs) have been developed. LNs have a low cost, enhance the water dispersion of EOs by many times, improve the penetration of EOs into plant tissues and control their release, and protect active compounds in EOs from adverse environmental conditions (Nguyen *et al.,* 2020). Bedoya-Serna *et al.* (2018) demonstrated that oregano oil-encapsulated LNs had minimal effective concentration against *Fusarium* sp. at an oregano oil concentration of 0.11µg/mL, while the native form (pure oil form) exhibited an effect against the fungus at a concentration of 150 µg/mL. Additionally, Hassanin *et al.* (2017) reported that LNs containing thyme oil and basil oil exhibited a significantly higher antifungal activity against *Fusarium oxysporum.* f. sp. *cumini* và *Fusarium oxysporum* than the native forms. Therefore, the minimal inhibitory concentration of EOs could be significantly reduced by encapsulating them into LNs, leading to a considerable reduction in the quantity and cost of EOs and enhancing their potential broad application as antifungal agents in green and sustainable agriculture.

Some recent studies have indicated that combinations of EOs result in significantly higher antifungal activities compared to the antifungal activities of single EOs due to the synergistic or additive effects (Bounar *et al.,* 2020; Park *et al.,* 2017; La-Torre *et al.,* 2016; Hossain *et al.,* 2016). For example, Park *et al.* (2017) demonstrated that the synergistic effect of origanum oil and thyme oil (ratio 1:1, v/v) significantly enhanced their inhibitory efficiency against mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* of 40%, 50% and 70% compared to origanum oil, thyme oil and untreated control, respectively. La-Torre *et al.* (2016) reported that combined treatment with rosemary oil, clove oil and thyme oil could reduce tomato Fusarium wilt much more efficiently in comparison with single treatment by clove oil, thyme oil, or rosemary oil. Moreover, EOs extracted from *Thymus vulgaris* and O*regano vulgare* exhibited a synergistic interaction to give a lower minimal inhibitory concentration and a higher antifungal activity against *Fusarium* spp. compared to the single forms (Bounar *et al.,* 2020).

The synergistic effect of EOs in a combined formulation has been demonstrated by using the fractional inhibition concentration index which was manually calculat-

ed (Bounar *et al.,* 2020; Hossain *et al.,* 2016). Meanwhile, the SynergyFinder software with the Bliss independent model was recently developed to demonstrate the synergistic/additive/antagonistic effect of drugs (Ianevski *et al.,* 2017), but it has not been intensively applied to investigate the synergistic/additive/antagonistic effect of EOs for the control of plant fungal pathogens. Only Nguyen *et al.* (2020) have successfully applied this software to demonstrate the synergistic effect of chili oil, cinnamon oil and neem oil (EO-LN forms) in nematode control. The application of the SynergyFinder software with the Bliss independent model to demonstrate the interaction (synergistic/additive/antagonistic effect) of EO components (the combined formulations) in the control of *F. incarnatum* will give the outcome of this study more scientific significance.

In the present research, the *in-vitro* antifungal activities of these three EO-LNs against *F. incarnatum* and of their combined binary and ternary formulations were assessed in laboratory bioassays. In addition, the synergistic/additive/antagonistic effects of these EOs in the combined formulations were determined using the SynergyFinder software with the Bliss independence model. Finally, the effect of the optimal EO-LN formulation on plant growth was evaluated. To our knowledge, the encapsulation of lemongrass (*Cymbopogon citratus*) oil (NaL), garlic (*Allium sativum*) oil (NaG) and mustard (*Sinapis alba*) oil (NaM) into LNs to control *F. incarnatum* has not been studied so far nor the antifungal activities of combined binary and ternary formulations of the EO-LNs.

Materials and Methods

Reagents and Biological Material

Garlic oil, mustard oil, and lemongrass oil were purchased from Dalosa Vietnam Essential – Herbal Co., LTD. (Vietnam). Soybean lecithin (Junsei Chemical, Tokyo, Japan) and Tween 80 (Samchun Pure Chemical, Seoul, Korea) were used as surfactants. A *F. incarnatum* strain was isolated and cultured at DaLat Nuclear Research Institute (Vietnam). Lettuce plants were purchased from a local market. All other chemicals were of analytical grade.

Preparation of EO-LNs

Three different EO-LNs corresponding to lemongrass oil (NaL), garlic oil (NaG) and mustard oil (NaM) were prepared using a combined method of homogenization and sonication (Nguyen *et al.,* 2020). Briefly, a mixture (10 g) of lecithin and Tween 80 (ratio 1:1, w/w) was added to distilled water (80 g) and stirred at 70°C for 45 min. Each EO (10 g) was then added into the surfactant mixture and homogenized using an Ultra Turrax T25 basic homogenizer (IKA, Japan) at 16000 rpm for 4 min. This was followed by sonication using an *Ultrasonic Processor VCX750 (Sonics & Materials, U.S.) at 20% for 3 min to form a nanodispersion of NaL, NaG and NaM.*

*T*he combined binary formulations were prepared by mixing two (2) EO-LNs with an equal volume (1:1, v/v), and the combined ternary formulation was prepared by mixing the three (3) EO-LNs at a ratio 1:1:1 (v/v/v).

Physical characterization of the EO-LNs and their binary and ternary combinations

The mean particle size, the polydispersity index (PDI) and the zeta potential of the 3 EO-LNs and of their combined binary and ternary formulations were determined using a Nano-ZS nano-size analyzer (Malvern, UK). Briefly, samples were diluted 100 times with distilled water. The resulting dispersion was then added to a polystyrene latex cell. The measurements were carried out at a temperature of 25°C and a detector angle of 173°C.

In-vitro antifungal activities of the EO-LNs and their binary and ternary combinations

The *in-vitro* antifungal activities against *F. incarnatum* of the EO-LNs and their binary and ternary combinations were evaluated in Petri dishes at laboratory bioassays (Kam-

su *et al.,* 2019). The fungus was grown on a PDA (potato dextrose agar) medium supplemented with the EO-LN formulations (single forms or combined forms) at dilutions of 250, 500, 600 and 750 times (corresponding to total EO-concentration of 0.4 g/L, 0.2 g/L, 0.1667 g/L and 0.1333 g/L). The control was a sample without addition of any EO-LN formulation. After 5 days, the growth of *F. incarnatum* in all treatments was evaluated and the mycelial diameter was measured. The antifungal activity was determined based on the mycelial growth inhibition (GI %) calculated using the following formula (1):

$$GI\ (\%) = \frac{D_c - D_t}{D_c} \times 100 \qquad (1)$$

Where D_c and D_t represent the mycelial growth diameter of the control and treated sample, respectively.

Evaluation of the effect of EO-LN formulation on plant growth

The effect of EO-LN formulation on plant growth (phytotoxicity) was investigated by spraying the optimal EO-LN formulation (determined in the previous section) at a high dilution concentration (250 times) on lettuce plants. Particularly, lettuce plants (112 plants/treatment) were grown in a greenhouse under natural light and at a room temperature of 22–28°C. When the plants had reached the stage of 3–4 leaves, the optimal EO-LN formulation was sprayed on the plants (about 250 mL/treatment). Spraying with water was used as a control. The experiment was repeated two more times. The average height of the plants at 3 and 7 days after treatment was determined, and the percentage of additional growth (AG %) was calculated using the formula (2):

$$AG\ (\%) = \frac{L_a - L_c}{L_c} \times 100 \qquad (2)$$

Where: L_a is the lettuce height after 7 days
L_c is the lettuce control height

Data analysis

One-way ANOVA with the post-hoc Duncan test on the SPSS software ($P < 0.05$) was used for statistical analysis. The synergistic/additive/antagonistic effects of NaL, NaG and NaM in their binary and ternary combinations were determined using the SynergyFinder software with the Bliss independence model. In this software, scores < –10 indicate that the interaction between two agents is likely antagonistic, scores from –10 to 10 indicate that the interaction between two agents is likely additive, and scores > 10 indicate that the interaction between two or three agents is likely to be synergistic (Ianevski *et al.,* 2017).

Results

Preparation of the EO-LNs

The garlic oil-encapsulated LNs (NaG), lemongrass oil-encapsulated LNs (NaL), and mustard oil-encapsulated LNs (NaM) had a mean particle size of 71.81 nm, 72.06 nm, and 138 nm, respectively. The polydispersity index (PDI) of these EO-LNs was less than 0.245 and their zeta potential was larger than -40 mV. The combined binary and ternary formulations of NaG, NaL and NaM had a small particle size less than 120 nm, a good PDI value of 0.180–0.245, and a high zeta potential more than -40 mV (Table 1).

As shown in Figure 1, the lemongrass oil, the garlic oil and the mustard oil were poorly dispersed in water (Figure 1A), while the dispersion of their corresponding EO-LNs in water was very high (Figure 1B). After the oils were encapsulated into LNs, the colors of the EO-LNs became lighter compared to the colors of the native EOs.

In-vitro antifungal activities of the EO-LNs and their binary and ternary combinations

The *in-vitro* antifungal activities against *F. incarnatum* of the three single formulations (NaM, NaG, NaL) and their combined formulations (NaMG, NaML, NaLG, NaMLG) were determined from their mycelial growth inhi-

bition (GI %) values, using bioassays in Petri dishes (Kamsu *et al.* 2019). The image in Figure 2 shows that the mycelium of *F. incarnatum* in the control sample grew fast with the largest growth diameter after incubation for 5 days, while the supplements of the EO-LN formulations in the PDA medium resulted in smaller mycelial growth diameters, corresponding to smaller GI values. As shown in Table 2, at 250 times dilution, all EO-LN formulations completely inhibited the growth of *F. incarnatum*. At 500 times dilution, the GI values of NaL, NaM, and NaG decreased from 100% to 20.78%, 32.55% and 43.53%, respectively, while the GI values of their binary (NaML, NaLG, NaMG) and ternary combinations (NaMLG) remained 100%. A further increase in the dilution concentration to 600 times resulted in a decrease in the GI values of NaML, NaMG and NaMLG from 100% to 33.33%, 56.08% and 22.35%, respectively. However, the GI value of NaLG was above 96% at this dilution concentration. At 750 times dilution, the GI values of the combined formulations sharply decreased to below 40% each, except for NaLG whose GI value became 62.35%.

When the synergistic/additive/antagonistic effects of NaL, NaG and NaM in their combined binary and ternary formulations were determined, the Bliss synergy scores (SynergyFinder software with the Bliss independence model) of the binary combinations of NaM and NaL, NaM and NaG, and NaL and NaG were 26.72, 19.05, and 22.37, respectively, while that of the ternary combination (NaMLG) was 100 (Figure 3).

Evaluation of the effects of EO-LN formulations on plant growth

Before being practically applied, the effect of optimal EO-LNs formulation on plant growth should be considered. Based on the *in-vitro* antifungal activities determined in the previous section, NaLG was chosen. Assssement of its effect on plant growth by spraying NaLG at 250 times dilution on lettuce plants, indicated that after treatment by NaLG for 3 and 7 days, all lettuce plants still developed normally, with a survival rate of 100%. In addition, there was no statistical difference in the percentage of additional growth between the treated and untreated plants (control) (Table 3). The heights of

Table 1. Physical characteristics of NaL, NaG and NaM and their combined binary, ternary formulations (n = 3).

EO-LNs formulations	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
NaM	138.00 ± 2.38a	0.174 ± 0.008d	-46.28 ± 1.29c
NaL	72.06 ± 1.45d	0.243 ± 0.012a	-41.37 ± 1.07ab
NaG	71.81 ± 1.51d	0.199 ± 0.013bc	-40.65 ± 1.31ab
NaM + NaL (NaML)	106.15 ± 2.28b	0.182 ± 0.011cd	-42.25 ± 0.93ab
NaM + NaG (NaMG)	107.10 ± 1.15b	0.241 ± 0.009a	-43.00 ± 1.12b
NaL + NaG (NaLG)	72.22 ± 1.21d	0.215 ± 0.012b	-40.10 ± 2.35a
NaM + NaL + NaG (NaMLG)	95.56 ± 1.74c	0.242 ± 0.011a	-41.48 ± 1.14ab

Binary combinations: NaML (NaM + NaL at a ratio 1:1, v/v); NaMG (NaM + NaG at a ratio 1:1, v/v); NaLG (NaL + NaG at a ratio 1:1, v/v). Ternary combination: NaMLG (NaM + NaL + NaG at a ratio 1:1:1, v/v/v).
Any two means followed by the same superscript letters in the same column are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

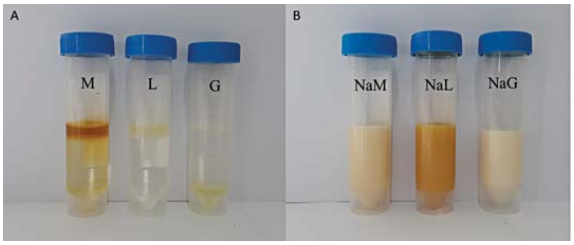

Figure 1. Water dispersion of (A) three Essential Oils (EOs) and (B) their corresponding Lipid Nanoemulsions (EO-LNs); M: mustard, L: lemongrass, G: garlic.

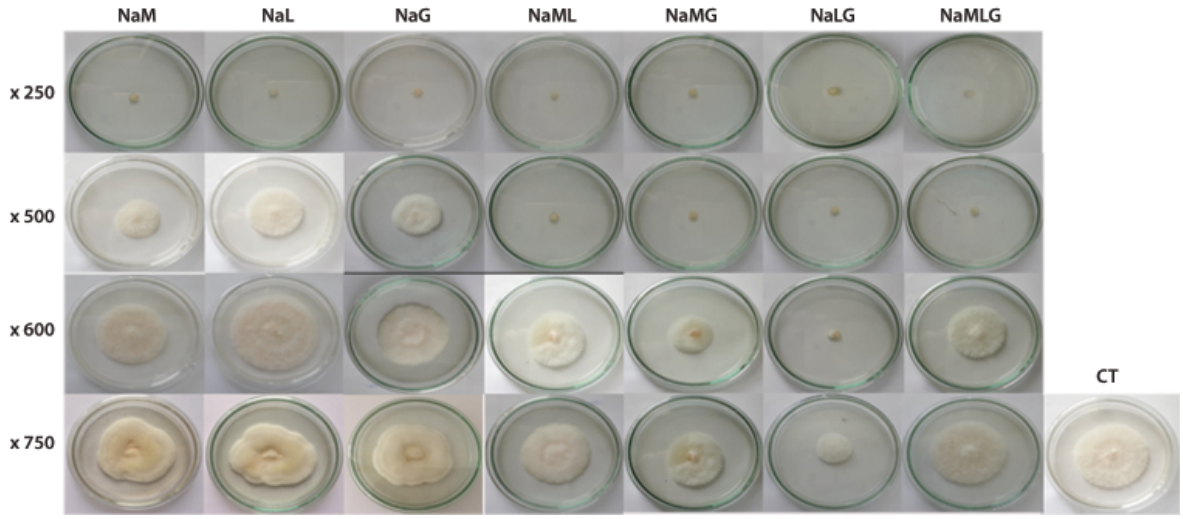

Figure 2. In-vitro antifungal activity against *Fusarium incarnatum* of NaL, NaG, NaM and their binary, ternary combinations. CT: control.

Table 2. *In-vitro* antifungal activity againts *Fusarium incarnatum* of nanoemulsions NaL (lemongrass), NaG (garlic), NaM (mustard) and their combined binary, ternary formulations.

Nanoformulation of essential oils	Growth inhibition (%)			
	250 times dilution	500 times dilution	600 times dilution	750 times dilution
NaM	100 ± 0a	32.55 ± 1.36c	5.88 ± 2.35e	0e
NaL	100 ± 0a	20.78 ± 3.59b	0.39 ± 1.36f	0e
NaG	100 ± 0a	43.53 ± 4.08d	2.75 ± 1.36ef	0e
NaML	100 ± 0a	100 ± 0a	33.33 ± 3.59c	13.73 ± 3.59c
NaMG	100 ± 0a	100 ± 0a	56.08 ± 2.72b	38.82 ± 2.35b
NaLG	100 ± 0a	100 ± 0a	96.08 ± 1.36a	62.35 ± 4.08a
NaMLG	100 ± 0a	100 ± 0a	22.35 ± 2.35d	7.45 ± 2.72d

Binary combinations: NaML (NaM + NaL at a ratio 1:1, v/v); NaMG (NaM + NaG at a ratio 1:1, v/v); NaLG (NaL + NaG at a ratio 1:1, v/v). Ternary combination: NaMLG (NaM + NaL + NaG at a ratio 1:1:1, v/v/v).
Any two means followed by the same superscript letters in the same column are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

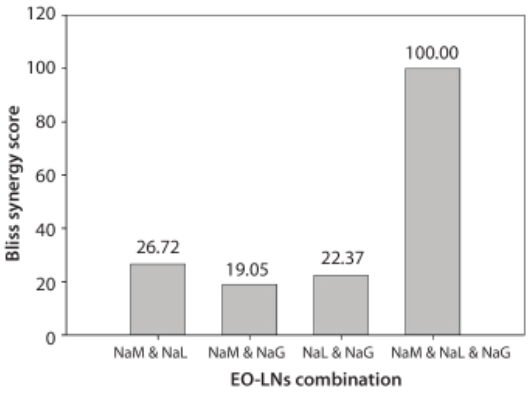

Figure 3. Bliss synergy score of binary and ternary combinations of NaL, NaG and NaM. The synergistic scores were determined by SynergyFinder software based on the results from three independent experiments.

lettuce plants in both cases increased by approximately 48% after 7 days.

Discussion

Based on their small mean particle sizes, good PDIs and high zeta potentials, it is clear that the EO-LNs of lemongrass, garlic and mustard were successfully prepared by a combined method of homogenization and sonication. The difference in the mean particle sizes of NaM, NaG, and NaL could be explained by the correlation between the RHLB

Table 3. Effect of NaLG at 250 times dilution on the growth of lettuce plants (n = 112).

Plant height / Sample	Before treatment (cm)	After 3 days (cm)	After 7 days (cm)	AG value (%)
Control	3.10 ± 0.21	3.47 ± 0.21	4.59 ± 0.29	47.97 ± 3.36a
Treated by NaLG at 250 times dilution	3.88 ± 0.32	4.53 ± 0.28	5.74 ± 0.38	47.94 ± 2.54a

Any two means followed by the same superscript letters in the column of AG value are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

(required hydrophilic–lipophilic balance) of each EO and the HLB (hydrophilic–lipophilic balance) of the surfactant mixture (Kim *et al.,* 2014). The high zeta potential values (above -40 mV) of the prepared EO-LNs indicate the high stability of these nanoformulations. The high stability can be explained by the fact that the high absolute value of the zeta potential (more than 30 mV) of the nanoparticles in the nanoformulations provided repulsive forces strong enough to prevent the particles from joining together to form larger particles (Mehnert *et al.,* 2001). In addition, the high dispersion of the EO-LNs in water compared to the dispersion of their corresponding oil form (native form) re-affirmed their successful formation. On the other hand, after equal volume ratios of the single EO-LNs were mixed to form combined binary or ternary formulations, the resulting formulations still maintained good physical characteristics nearly similar to those of their "parent" single formulations. In addition, no strange phenomena such as creaming, precipitation or chemical reaction occurred. These indicated that it is possible to prepare combined binary and ternary formulations from NaL, NaG and NaM.

Previous studies have reported high antibacterial and antifungal activities of garlic oil, lemongrass oil and mustard oil (Kamsu *et al.,* 2019; Roselló *et al.,* 2015; Wang *et al.,* 2019). However, as mentioned before, the antifungal activities of these EOs against *Fusarium sp.* are not so strong and usually require a high EO concentration to exhibit an obvious effect (Drakopoulos *et al.*, 2020; Kamsu *et al.*, 2019; Sharif *et al.*, 2017; Hayat *et al.*, 2016). For example, Kamsu *et al.* (2019) showed that lemongrass oil (density of 0.893 g/mL) only moderately inhibited mycelial growth of *F. incarnatum* at a concentration of more than 500 µL/L (approximately 0.4465 g/L). Hayat *et al.* (2016) reported that an obvious inhibition against *F. oxysporum* of garlic extract was achieved at allicin concentration of approximately 3.9 g/L (10 g garlic homogenized in 100 mL distilled water). In addition, Drakopoulos *et al.* (2020) also showed that mustard seed powders (containing more than 28-32% oil) could inhibit the mycelial growth of *Fusarium graminearum* at a high concentration of 20 g/L, corresponding to an approximately mustard oil concentration of 6 g/L (Drakopoulos *et al.*, 2020; Sharif *et al.*, 2017).

In this study, based on the GI values, it is clear that all three EO-LNs had strong antifungal activities against *F. incarnatum*; each one had a GI value of 100% at 250 times dilution (corresponding to an EO concentration of approximately 0.4 g/L). In addition, the GI values at 500 times dilution indicated that the antifungal activity of NaG against this fungal strain was the highest compared to those of NaL and NaM. Moreover, the results also indicated that the binary and ternary combinations of these single EO-LNs were considerably more effective against *F. incarnatum*, with GI values of 100% each at 500 times dilution (corresponding to concentrations of 0.1 g/L and 0.0667 g/L for each EO in the combined binary formulations and combined ternary formulation, respectively). The antifungal results at 600 times dilution showed that the binary combination of NaL and NaG at a ratio 1:1 (v/v) (NaLG) had the highest antifungal activity of all binary combinations. This nanoformulation nearly completely inhibited the growth of *F. incarnatum* (GI value of 96.08%) at a concentra-

tion of 0.0833 g/L for each constituent EO. This EO concentration was many times lower than that obtained by Kamsu *et al.* (2019). This could be explained by the synergistic effect of NaL (lemongrass oil) and NaG (garlic oil), as indicated by a Bliss syngergy score of 22.37 calculated by the SynergyFinder software with the Bliss independence model.

The ternary combination of the EOs had a higher Bliss syngergy score than that of the binary combinations, which indicates a higher synergistic effect. More specifically, although the concentration of each constituent EO of the binary formulation at 750 times dilution was 0.0667 g/L and equalled to that of the ternary formulation at 500 times dilution, the GI value of NaMLG reached 100%, compared to 13.73%, 38.82% and 62.35% of NaML, NaMG and NaLG, respectively. This has illustrated that the synergistic effect of ternary combination resulted in higher antifungal acitivity against *F. incarnatum* than the binary combinations. This is similar to the result reported in our previous study, where the ternary combination of chili oil-encapsulated LNs, garlic oil-encapsulated LNs and cinnamon oil-encapsulated LNs also exhibited the highest *in-vitro* antifungal activity against *Alternaria alternata* due to their synergistic effect (Nguyen *et al*., 2022). It has also been reported that the ternary combination of chili oil-, cinnamon oil- and neem oil-encapsulated LNs exhibited higher nematocidal activity than their combined binary formulations (Nguyen *et al.,* 2020). Moreover, a study of Jyoti *et al.* (2019) also revealed that the ternary combination of clove oil (*Syzygium aromaticum*), cinnamon oil (*Cinnamomum zeylanicum*), and lemongrass oil (*Cymbopogon citratus*) at a ratio of 1:1:1 (v/v/v) had higher synergistic efficacy than the binary combinations in controlling *Rhipicephalus microplus*.

Nevertheless, at 600 times dilution, the binary combination of NaL and NaG (NaLG) exhibited higher antifungal activity compared to the ternary combination (NaMLG) in the same dilution. This could be explained by two reasons. First, the concentration of each constituent EO of the ternary formulation at 600 times dilution was only 0.0556 g/L, while that of the binary formulation was 0.0833 (approximately 1.5 times higher). Secondly, at this dilution, the low concentration (0.0556 g/L) of lemongrass oil, garlic oil and mustard oil contained in the ternary formulation might be lower than the minimal inhibitory concentration (MIC) needed to effectively inhibit the growth of *F. incarnatum* (Dutta *et al*., 2021; Eke *et al*., 2020; Wang *et al*., 2020). Particularly, MIC for controlling *Fusarium solani* of lemongrass oil was 0.5 g/L (Eke *et al*., 2020), while MIC against *Fusarium oxysporum* f. sp. *lycopersici* of mustard oil was 0.256 g/L (Dutta *et al*., 2021). In addition, MIC against *F. oxysporum* and *Fusarium solani* of garlic oil was approximately 0.05 g/L (Wang *et al*., 2020). On the other hand, the combination of different EOs (or EO-LNs) can also limit the resistance of fungal strains compared to the use of single-EO (or EO-LNs) formulations (Hossain *et al.,* 2016). From all above results and discussion, it is possible to conclude that NaLG was the best formulation for controlling *F. incarnatum*.

Furthermore, NaLG did not negatively affect the growth of lettuce plants, even at the high total EO concentration of 0.4 g/L (corresponding to 250 times dilution). None of the symptoms of chlorosis, cell death and reduced growth was observed during the experiment. This might be because after spraying on plants, nano-carriers (NaLG) had deeply penetrated into the plant tissues and slowly released EOs thus maintaining the concentration of EOs in the plant tissue at a level lower than the minimal inhibition concentration (Rehman *et al.,* 2021). Moreover, sulfur compounds in garlic have also been reported to be able to promote plant growth (Cheng *et al.,* 2016).

Conclusion

NaM, NaL, and NaG were successfully prepared by a combined method of homogenization and sonication with good physical charateristics (small mean particle size, small PDI and high zeta potential). Their combined

binary and ternary formulations were also successfully prepared by mixing with the equal volume ratio of each EO-LNs. The *in-vitro* antifungal activity of NaG against *F. incarnatum* was higher than those of NaL and NaM. In addition, the combined binary, ternary formulations exhibited higher antifungal activity than the single formulations due to the synergistic effect as determined by the SynergyFinder software with the Bliss independence mode. The results also demonstrated that, at the same concentration of each constituent EO, the ternary combination had higher antifungal activity compared to the binary combinations. However, at 600 times dilution, the antifungal activity against *F. incarnatum* of NaLG (binary combination) achieved the highest GI value (more than 96%), which was significantly higher than that of NaMLG (ternary combination), possibly due to the 1.5-time higher concentration of each constituent EO compared to that of the ternary formulation. In addition, NaLG at 250 times dilution (corresponding to total EO concentration of 0.4 g/L) did not negatively affect the growth of lettuce plants. Therefore, NaLG has the potential to be widely applied in sustainable agriculture to protect plants from diseases caused by *F. incarnatum*.

This study was partly supported by Institute of Developmental Philosophy (Vietnam) and Nuclear Research Institute (Vietnam). We also would like to thank student Minh-Trong Le for his contribution in this study.

Declarations

Conflicts of interest/Competing interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Literature cited

Akram, W., Ahmad, A., Luo, W., Yasin, N.A., Wu, T., Guo, J., Wang, Q. and Li, G. 2019. First report of stem and root rot of Chinese kale caused by *Fusarium incarnatum-equiseti* species complex in China. *Plant Disease*, 103: 1781. DOI: https://doi.org/10.1094/PDIS-02-19-0261-PDN.

Amini, M., Safaie, N., Salmani, M.J. and Shams-Bakhsh, M. 2012. Antifungal activity of three medicinal plant essential oils against some phytopathogenic fungi. *Trakia Journal of Sciences,* 10: 1–8.

Bedoya-Serna, C.M., Dacanala, G.C., Fernandes, A.M. and Pinho, S.C. 2018. Antifungal activity of nanoemulsions encapsulating oregano (*Origanum vulgare*) essential oil: *in vitro* study and application in Minas Padrão cheese. *Brazilian Journal of Microbiology,* 49: 929–935. DOI: https://doi.org/10.1016/j.bjm.2018.05.004.

Boukhatem, M.N., Ferhat, M.A., Kameli, A., Saidi, F. and Kebir, H.T. 2014. Lemongrass (*Cymbopogon citratus*) essential oil as a potent anti-inflammatory and antifungal drugs. *Libyan Journal of Medicine,* 9: 25431 (p. 1–10). DOI: https://doi.org/10.3402/ljm.v9.25431.

Bounar, R., Krimat, S., Boureghda, H. and Dob, T. 2020. Chemical analyses, antioxidant and antifungal effects of oregano and thyme essential oils alone or in combination against selected *Fusarium* species. *International Food Research Journal,* 27: 66–77.

Cheng, F., Cheng, Z., Meng, H. and Tang, X. 2016. The garlic allelochemical diallyl disulfide affects tomato root growth by influencing cell division, phytohormone balance and expansin gene expression. *Frontiers in Plant Science,* 9: 1199 (p. 1–16). DOI: https://doi.org/10.3389/fpls.2016.01199.

Drakopoulos, D., Meca, G., Torrijos, R., Marty, A., Kägi, A., Jenny, E., Forrer, H.R., Six, J. and Vogelgsang, S. 2020. Control of *Fusarium graminearum* in wheat with mustard-based botanicals: From *in vitro* to *in planta*. *Frontiers in Microbiology,* 11: 1595 (p. 1–15). DOI: https://doi.org/10.3389/fmicb.2020.01595.

Dutta, A., Mandal, A., Kundu, A., Malik, M., Chaudhary, A., Khan, M.R., Shanmugam, V., Rao, U., Saha, S., Patanjali, N., Kumar, R., Kumar, A., Dash, S., Singh, P.K. and Singh, A. 2021. Deciphering the behavioral response of *Meloidogyne incognita* and *Fusarium oxysporum* toward mustard essential oil. *Frontiers in Plant Science*, 12: 714730 (p. 1–16). DOI: https://doi.org/ 10.3389/fpls.2021.714730.

Eke, P., Adamou, S., Fokom, R., Wakam, L.N., Nwaga, D. and Boyom, F.F. 2020. Arbuscular mycorrhizal fungi alter antifungal potential of lemongrass essential oil against *Fusarium solani*, causing root rot in common bean (*Phaseolus vulgaris L.*). *Heliyon*, 6: e05737 (p. 1–9). DOI: https://doi.org/10.1016/j.heliyon.2020.e05737.

Gressel, J. 2020. Perspective: present pesticide discovery paradigms promote the evolution of resistance – learn from nature and prioritize multi-target site inhibitor design. *Pest Man-*

agement Science, 76: 421–425. DOI: https://doi.org/10.1002/ps.5649.

Hassanin, M.M.H., Abd-El-Sayed, M.A. and Abdallah, M.A. 2017. Antifungal activity of some essential oil emulsions and nanoemulsions against *Fusarium oxysporum* pathogen affecting cumin and geranium plants. *Scientific Journal of Flowers and Ornamental Plants,* 4: 245–258. DOI: https://doi.org/10.21608/sjfop.2017.11326.

Hayat, S., Cheng, Z., Ahmad, H., Ali, M., Chen, X. and Wang, M. 2016. Garlic, from remedy to stimulant: Evaluation of antifungal potential reveals diversity in phytoalexin allicin content among garlic cultivars; allicin containing aqueous garlic extracts trigger antioxidants in cucumber. *Frontiers in Plant Science,* 7: 1235 (p. 1–15). DOI: https://doi.org/10.3389/fpls.2016.01235.

Hossain, F., Follett, P., Dang, V.K., Harich, M., Salmieri, S. and Lacroix, M. 2016. Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food. *Food Microbiology,* 53 (Pt B): 24–30. DOI: https://doi.org/10.1016/j.fm.2015.08.006.

Ianevski, A., He, L., Aittokallio, T. and Tang, J. 2017. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. *Bioinformatics,* 33: 2413–2415. DOI: https://doi.org/10.1093/bioinformatics/btx162.

Jiang, N., Yan, J., Liang, Y., Shi, Y., He, Z., Wu, Y., Zeng, Q., Liu, X. and Peng, J. 2020. Resistance genes and their interactions with bacterial blight/leaf streak pathogens (*Xanthomonas oryzae*) in rice (*Oryza sativa* L.) - an updated review. *Rice,* 13: 3 (p. 1–12). DOI: https://doi.org/10.1186/s12284-019-0358-y.

Jyoti, Singh, N.K., Singh, H., Mehta, N. and Rath, S.S. 2019. *In vitro* assessment of synergistic combinations of essential oils against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Experimental Parasitology,* 201: 42–48. DOI: https://doi.org/10.1016/j.exppara.2019.04.007.

Kamsu, N.P., Tchinda, S.E., Tchameni, N.S., Jazet, D.P.M., Madjouko, M.A., Youassi-Youassi, O., Sameza, M.L., Tchoumbougnang, F. and Menut, C. 2019. Antifungal activities of essential oils of cinnamon (*Cinnamomum zeylanicum*) and lemongrass (*Cymbopogon citratus*) on crown rot pathogens of banana. *Indian Phytopathology,* 72: 131–137. DOI: https://doi.org/10.1007/s42360-018-0104-1.

Kim, J.H., Ko, J.A., Kim, J.T., Cha, D.S., Cho, J.H., Park, H.J. and Shin, G.H. 2014. Preparation of a capsaicin-loaded nanoemulsion for improving skin penetration. *Journal of Agricultural and Food Chemistry,* 62: 725–732. DOI: https://doi.org/10.1021/jf404220n.

La-Torre, A., Caradonia, F., Matere, A. and Battaglia, V. 2016. Using plant essential oils to control Fusarium wilt in tomato plants. *European Journal of Plant Pathology,* 144: 487–496. DOI: https://doi.org/10.1007/s10658-015-0789-2.

Li, Y., Nie, Y., Zhou, L., Li, S., Tang, X. and Ding, Y. 2014. The possible mechanism of antifungal activity of cinnamon oil against *Rhizopus nigricans*. *Journal of Chemical and Pharmaceutical Research,* 6: 12–20.

Mehnert, W. and Mäder, K. 2001. Solid lipid nanoparticles: production, characterization and applications. *Advanced Drug Delivery Reviews,* 47: 165–196. DOI: https://doi.org/10.1016/s0169-409x(01)00105-3.

Nguyen, M.H., Vu, N.B.D, Nguyen, T.H.N, Tran, T.N.M, Le, H.S., Tran, T.T, Le, X.C, Le, V.T, Nguyen, N.T.T. and Trinh, N.A. 2020. Effective biocontrol of nematodes using lipid nanoemulsions co-encapsulating chili oil, cinnamon oil and neem oil. *International Journal of Pest Management.* DOI: https://doi.org/10.1080/09670874.2020.1861361.

Nguyen, M.H., Tran, T.N.M. and Vu, N.B.D. 2022. Antifungal activity of essential oil-encapsulated lipid nanoemulsions formulations against leaf spot disease on tomato caused by *Alternaria alternata*. *Archives of Phytopathology and Plant Protection*, 55: 235–257. DOI: https://doi.org/10.1080/03235408.2021.2015887.

Omar, M.S. and Kordali, S. 2019. Review of essential oils as antifugal agents for plant fungal diseases. *Ziraat Fak Derg,* 14: 294–301.

Omidbeygi, M., Barzegar, M., Hamidi, Z. and Nafhdibadi, H. 2007. Antifungal activity of thyme, summer savory and clove essential oils against *Aspergillus flavus* in liquid medium and tomato paste. *Food Control,* 18: 1518–1523. DOI: https://doi.org/10.1016/j.foodcont.2006.12.003.

Park, J.Y., Kim, S.H., Kim, N.H., Lee, S.W., Jeun, Y.C. and Hong, J.K. 2017. Differential inhibitory activities of four plant essential oils on *in vitro* growth of *Fusarium oxysporum* f. sp. *fragariae* causing *Fusarium* wilt in strawberry plants. *Plant Pathology Journal,* 33: 582–588. DOI: https://doi.org/10.5423/PPJ.OA.06.2017.0133.

Peng, C., Zhao, S.Q., Zhang, J., Huang, G.Y., Chen, L.Y. and Zhao, F.Y. 2014. Chemical composition, antimicrobial property and microencapsulation of Mustard (*Sinapis alba*) seed essential oil by complex coacervation. *Food Chemistry,* 165: 560–568. DOI: https://doi.org/10.1016/j.foodchem.2014.05.126.

Rao, B.R.R., Kaul, P.N., Syamasundar, K.V. and Ramesh, S. 2005. Chemical profiles of primary and secondary essential oils of palmarosa (*Cymbopogon martinii* (Roxb.) Wats var. *motia* Burk.). *Industrial Crops and Products,* 21: 121–127. DOI: https://doi.org/10.1016/j.indcrop.2004.02.002.

Rehman, A., Qunyi, T., Sharif, H.R., Kormac, S.A., Karim, A., Manzoor, M.F., Mehmood, A., Iqbal, M.W., Raza, H., Ali, A. and Mehmood, T. 2021. Biopolymer based nanoemulsion delivery system: An effective approach to boost the antioxidant potential of essential oil in food products. *Carbo-*

hydrate Polymer Technologies and Applications, 2: 100082 (p. 1–12). DOI: https://doi.org/10.1016/j.carpta.2021.100082.

Roselló, J., Sempere, F., Sanz-Berzosa, I., Chiralt, A. and Santamarina, M.P. 2015. Antifungal activity and potential use of essential oils against *Fusarium culmorum* and *Fusarium verticillioides*. *Journal of Essential Oil Bearing Plants,* 18: 359–367. DOI: https://doi.org/10.1080/0972060X.2015.1010601.

Sharif, R.H., Paul, R.K., Bhattacharjya, D.K. and Ahmed, K.U. 2017. Physical characters of oil seeds from selected mustard genotypes. *Journal of Bangladesh Agricultural University,* 15: 27–40. DOI: https://doi.org/10.3329/jbau.v15i1.33527.

Wang, A., Bi, Y., Wen, X., Wang, Y. and Cai, H. 2020. Antifungal activity of 4 kinds of aromatic essential oil derived from plants to pathogenic fungi of bamboo. Scientia Silvae Sinicae, 56: 59–67. DOI: https://doi.org/ 10.11707/j.1001-7488.20200606.

Wang, Y., Wei, K., Han, X., Zhao, D., Zheng, Y., Chao, J., Kong, F. and Zhang, C.S. 2019. The antifungal effect of garlic essential oil on *Phytophthora nicotianae* and the inhibitory component involved. *Biomolecules,* 9: 632 (p. 1–12). DOI: https://doi.org/10.3390/biom9100632.

Yang, L.N., He, M.H., Ouyang, H.B., Zhu, W., Pan, Z.C., Sui, Q.J., Shang, L.P. and Zhan, J. 2019. Cross-resistance of the pathogenic fungus *Alternaria alternata* to fungicides with different modes of action. *BMC Microbiology,* 19: 205 (p. 1–10). DOI: https://doi.org/10.1186/s12866-019-1574-8.

Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R. and Schäfer, R.B. 2019. Fungicides – an overlooked pesticide class?. *Environmental Science & Technology,* 53: 3347–3365. DOI: https://doi.org/10.1021/acs.est.8b04392.

Received: 22 September 2021; Accepted: 28 September 2022

Αξιολόγηση της *in-vitro* αντιμυκητιακής δράσης έναντι του *Fusarium incarnatu*m, σκευασμάτων σε μορφή νανογαλακτώματος με ενθυλάκωση δύο ή τριών αιθέριων ελαίων λεμονόχορτου, σκόρδου και λευκού σιναπιού

Minh-Hiep Nguyen και Thi-Ngoc-Mai Tran

Περίληψη Νανογαλακτώματα με ενθυλάκωση αιθέριων ελαίων σκόρδου, λεμονόχορτου και λευκού σιναπιού παρασκευάστηκαν με συνδυασμένη μέθοδο ομογενοποίησης και υπερήχων με στόχο τη δημιουργία αποτελεσματικών σκευασμάτων κατά του *Fusarium incarnatum* (εργαστηριακές βιοδοκιμές). Επίσης δοκιμάστηκαν διμερείς και τριμερείς συνδυασμοί τους (παρασκευάσματα με ανάμειξη ίσης αναλογίας όγκου κάθε αιθέριου ελαίου). Η συνεργιστική/αυξανόμενη/ανταγωνιστική αντιμυκητιακή δράση των νανογαλακτωμάτων στις συνδυασμένες συνθέσεις των αιθέριων ελαίων προσδιορίστηκε με τη χρήση του λογισμικού SynergyFinder και το μοντέλο ανεξαρτησίας Bliss. Τα αποτελέσματα έδειξαν συνεργιστική δράση για τους διμερείς και τριμερείς συνδυασμούς νανο σκευασμάτων των αιθέριων ελαίων σκόρδου, λεμονόχορτου και λευκού σιναπιού. Επιπλέον, ο τριμερής συνδυασμός αιθέριων ελαίων, με συμμετοχή κάθε συστατικού στην ίδια συγκέντρωση, είχε υψηλότερη αντιμυκητιακή δράση από τους διμερείς συνδυασμούς. Ωστόσο, το νανογαλάκτωμα του συνδυασμού αιθέριων ελαίων σκόρδου-λεμονόχορτου, με αραίωση 600 φορές, ανέστειλε κατά 96% τη μυκηλιακή ανάπτυξη του *F. incarnatum,* αποτελεσματικότητα που ήταν σημαντικά υψηλότερη από αυτή του συνδυασμού των τριών αιθέριων ελαίων στην ίδια αραίωση. Αυτό θα μπορούσε πιθανώς να αποδοθεί στην κατά 1,5 φορά υψηλότερη συγκέντρωση κάθε συστατικού αιθέριου ελαίου στον διμερή συνδυασμό σε σύγκριση με εκείνη στον τριμερή. Επιπλέον, το νανογαλάκτωμα του συνδυασμού αιθέριων ελαίων σκόρδου-λεμονόχορτου, ακόμα και στην υψηλή συγκέντρωση αιθέριων ελαίων των 0,4 g/L, δεν προκάλεσε συμπτώματα φυτοτοξικότητας σε φυτά μαρουλιού.

Hellenic Plant Protection Journal **16:** 1-11, 2023

sciendo

Hellenic Plant Protection Journal **16:** 12-22, 2023
DOI 10.2478/hppj-2023-0002

Unraveling the role of endophytic fungi in barley salt-stress tolerance

R. Kouadria[1,2*], M. Bouzouina[2], B. Lotmani[2] and S. Soualem[3]

Summary Salinity is an agricultural and eco-environmental problem worldwide that decreases crop production. Endophytic fungi have been shown to improve plant tolerance to stressful conditions. The purpose of the paper is to examine the efficiency of *Embellisia phragmospora*, *Fusarium equiseti* and *Fusarium graminearum* to improve tolerance of pot-grown barley in greenhouse under different levels of soil salinity (2.5, 8 and 14dS/m) by estimating growth, relative water content, mineral nutrition, photosynthetic pigments biosynthesis, proline and sugar levels. Results showed that *E. phragmospora* increased barley emergence rate to 66.7% compared to 60% recorded by non-colonized barley under 14dS/m soil salinity. The tested endophytes increased barley root length, shoot and root dry weights under salt stress. Endophytic fungi reduced Na^+ accumulation and improved K^+ uptake in barely under salinity. *Fusarium equiseti* and *F. graminearum*-inoculated barley increased proline content under salinity. *Fusarium graminearum*-colonized barley showed the highest sugar content under salt stress. Our findings demonstrate the feasibility of endophytic fungi bio-inoculation in improvement of barley tolerance to salt stress, which qualify them to be a potent tool to provide substantial benefits to crops for sustainable agriculture.

Additional keywords: Barley, Bio-inoculation, Endophytic fungi, Salt stress, Tolerance

Introduction

Among the abiotic stresses, salinity is one of the major problems that affect crop growth and development (Ruiz-Lozano *et al*., 2012). Wang *et al*. (2003) suggested that increased salinity of agricultural land is expected to have damaging effects, resulting in 30% land loss within the next 25 years and up to 50% before the end of 21st century.

Salt-stressed plants suffer from K^+ deficiency and Na^+ toxicity which disrupts photosynthesis, enzymes activity, protein biosynthesis (Gupta and Huang, 2014) and accumulation of osmolytes, such as proline and sugars (Majumder *et al*., 2010).

Barley (*Hordeum vulgare* L.), the most important cereal crop in the world, is considered to be moderately salt sensitive (Munns *et al*., 2006). Barley yield damages in saline areas are significant; therefore salt stress tolerance is a widely sought quality in order to extend its cultivation to unfavorable regions (Baenziger *et al*., 2006).

Desalination and deployment of salt tolerant barley varieties can both be used to combat salinity stress. However, development of salt tolerant varieties through traditional breeding or advanced molecular techniques is time consuming and highly expensive. Quite recently, considerable attention has been paid to identification of alternative methods to enhance plant productivity under abiotic stresses (Wei and Jousset, 2017). Endophytic fungi have been gaining importance in recent years to explain their role in reducing negative effects of environmental stresses, such as salinity (Aghilia *et al*., 2014).

Munns and Tester (2008) showed that phytohormones are regulated by signaling genes involved in salt and osmotic stress alleviation under salt stress conditions. Leitão and Enguita (2016) have demonstrated that endophytic fungi can synthesize phytohormones such as gibberellins, auxins (IAA) and

[1] Higher School of Agronomy, Kharrouba-Mostaganem 27000.
[2] Plant Protection Laboratory, Abdelhamid IBN Badis - Mostaganem University, Kharrouba, 27000 Mostaganem, Algeria.
[3] Ibn Khaldoun University - Tiaret 14000.
* Corresponding author: rabia-rk@hotmail.com
ORCID: https://orcid.org/0000-0003-2632-0937

abscisic acid (ABA) to improve plant tolerance under stressful conditions. As reported by Hasan (2002) the gibberellin produced by *Fusarium* spp. under salt stress conditions can reduce the negative effect of salinity and promote plant growth.

One of the examples of the feasibility of endophytic fungi to increase plant tolerance to salt stress was presented by Gill *et al*. (2016) reporting that the endophytic basidiomycete, *Piriformospora indica*, can colonize plants roots and improve plant tolerance under salinity. Furthermore, it has been shown that *Piriformospora indica* avoids Na^+ uptake and its translocation to leaves and increases K^+ uptake under salt stress (Yun *et al*., 2018). Endophytic fungi increased proline (Yang *et al*., 2004) and sugar content (Robert-Seilaniantz *et al*., 2007) as an osmoprotectant under salt stress conditions.

Positive impact of endophytes on wheat plants under salt stress conditions was evaluated, *in vitro*, in our previous study (Kouadria *et al*., 2018). The study showed that endophytic fungi improved wheat seedlings germination and growth under saline conditions (400, 600 and 800 meq/l NaCl) compared to non-inoculated plants where germination was inhibited under 600 and 800 meq/l NaCl. Moreover, the endophytic fungus *Chaetomium coarctatum* has been shown to improve salt stress tolerance of barley plants (Kouadria *et al*., 2020).

The paper aims to give comprehensive account of the ability of endophytic fungi to improve barley agricultural traits under salt stress. The ability of endophytic fungi to increase barley salt stress tolerance was assessed through measuring growth, relative water content, photosynthetic pigments biosynthesis, mineral nutrition and osmoregulation under both saline and non-saline conditions.

Materials and Methods

Spore suspension preparation

Fungi strains used in this research, *Embellisia phragmospora*, *Fusarium equiseti* and *Fusarium graminearum*, were isolated from *Beta macrocarpa* Guss., *Salsola oppositifolia* Desf., and *Lolium rigidum* Gaudin, respectively (Kouadria *et al*., 2019).

Isolates were allowed to grow and sporulate on PDA medium. Cultures were incubated at 28-30°C for 7 days. Spores were scraped off from the agar surface with distilled water and suspended in 0.05% Tween 80 solution. The spore suspension was then collected in a sterile test tube and shaken for 2 min. The suspension was filtered through a double layered mesh serially diluted and its concentration was determined microscopically with Hemocytometer (Akagi *et al*., 2015).

Endophytes-barley association – Experimental set up

Barley (*Hordeum vulgare* var. Saida 183) seeds were surface sterilized (5min) by sodium hypochlorite solution (NaClO, 5%) and distilled water. Then, seeds were inoculated by immersion in fungi spore suspensions (10^7 spores / mL) for 24 h. Non-inoculated seeds (control) were placed in distilled water. Seeds were pre-germinated in phytotron at 25°C for 24 h without substrate.

Soils used in the experiment are naturally salty soils, and their electrical conductivity was calculated by the National Institute of Soils, Irrigation and Drainage, El Matmar, Relizane, Algeria.

Pre-germinated seedlings were transferred to pots (19 cm in diameter and 50 cm in length), filled with autoclaved soil; unsalted soil with EC=2.5 dS/m, moderate and high saline soil with EC=8 dS/m and EC=14 dS/m, respectively. In each pot ten seedlings were planted with three replicates for each treatment (10 seedlings/pot and 3 pots/treatment). The treatments comprised controls and endophyte-inoculated wheat plants under non-saline conditions (2.5dS/m), controls for salt stress (8 and 14dS/m), and endophyte-inoculated plants under salt stress conditions (8 and 14dS/m). Measurements of EC were conducted at the end of the tests to confirm that soil salinity had not changed after the treatment period. The experimen-

tal design was randomized complete block with four sets of treatments. The experiment was carried out under greenhouse conditions with 12 h light, relative humidity of 55–65% and 25/18 °C (day/night).

Measurements

Growth parameters

Barley seedling emergence rate was estimated 15 days after treatment. After 90 days of growth under salt treatments, barley seedlings were harvested at the heading stage. Root length and biomass production (root and shoot dry weights were measured after drying in oven 75°C for 72 h) were measured. Leaf area was calculated according to Li et al. (2004) using the following equation (5leaves/treatment):

$$LA\ (cm^2) = (MLL+MLW) \times 0.75$$

MLL: maximum leaf length. MLW: maximum leaf width.

Relative water content (RWC)

The RWC of each leaf was calculated according to Scippa *et al*. (2004) using the following equation:

$$RWC\ (\%) = [(FW-DW)/(TW-DW)]\times 100$$

where: FW is the fresh weight of the sampled leaf tissue. TW is the turgid weight of a leaf soaked in distilled water overnight at 4°C, and DW is the dry weight of that leaf after 48 h at 80°C.

Mineral nutrients

Potassium (K^+) and sodium (Na^+) contents were measured using diacid method (nitric acid and hydrochloric acid) according to Chapman and Pratt (1961). Leaves were dried in an oven at 80° C for 48 hours and then ground. After cooling, 50 mg of dry matter were placed in crucibles, and then put in a muffle oven at 600° C for 6 h. After cooling, the recovered ashes were subjected to an acid attack in 4 mL of 35% nitric acid, and the crucibles were placed on a sand bath until the organic matter was completely dissolved. After complete solvent evaporation, the mineral elements were suspended in 10 mL of 0.1 mol l^{-1} hydrochloric acid (HCl) and filtered with Wattman # 1 filter paper. Results were obtained after flame photometry analysis (model PFP7; Jenway, Stone, UK).

Chlorophyll and carotenoids content

Chlorophyll a (Chlo a), chlorophyll b (Chlo b), total chlorophyll (Chlo T) and carotenoids (CART), of fresh fully-expanded leaves were determined as described by Arnon (1949). Fresh leaf samples (0.1 g) were homogenized in a mortar and extracted with 5 mL acetone 80 % solution using Whatman No. 42 filter paper. The absorbance of the extract was recorded at 470 (A470), 663 (A663) and 645 (A645) nm wavelengths using a Jenway 67155 UV/Vis spectrophotometer.

Soluble sugar content

Soluble sugars were measured by the method of Schields and Burnett (1960). Fresh leaf tissue (100mg) was added to tubes containing 5.25 mL of ethanol 80% for 24 hours. The extract obtained was diluted 10 times with ethanol 80%. To 2 mL of the homogenate, 4 mL of anthrone reagent (2 g anthron + 1000 mL sulfuric acid (H_2SO_4)) was added. The anthrone reagent must be prepared 4 h before carrying out the tests. The homogenate was delicately mixed and kept in melting ice. Then the mixture was boiled in water bath at 92° C for 8 min, and then cooled for 30 minutes in the dark. Absorbance was measured at 585 nm. A standard curve was established using glucose and results are therefore expressed in mg/g of fresh weight (FW).

Proline content

The method of Troll and Lindsley (1955) was carried out to monitor the proline content. Fresh leaf tissues (100 mg) were homogenized in 2 mL methanol 40%. To 2 mL of homogenate, 2 mL of acid ninhydrin (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL of 6 mol l^{-1} phosphoric acid) and 2 mL of glacial acetic acid were added. The mixture was boiled in a water bath at 100°C for 60 min. The boiled mixture was then toluenized with 5 ml of toluene. Ab-

sorbance was measured at 528 nm. Proline concentration was determined by following a calibration curve and expressed as mg/g of fresh weight (FW).

Data analysis

Experiments comprised of 10 plants per pot, while each treatment comprised of three replicates. Data were analyzed by means of 2-way ANOVA with 2 factors (fungal inoculation * salt concentration). For each trait an analysis of variance (ANOVA) was performed to compare endophyte-colonized plants in each treatment to their endophyte-free counterparts. Each ANOVA was followed by a post hoc Fischer's least significant difference (LSD) test. P-values less than alpha levels of 0.05 were considered significant. Statistical tests were run using Statbox v6.4 statistical software.

Results and discussion

Salinity is one of the critical abiotic factors that severely disturb plant growth and metabolism (Deinlein *et al*., 2014). Barley is an important cereal cultivated worldwide. However, its production is under threat due to ever increasing environmental stresses including salinity (Baenziger *et al*., 2006).

The results showed that salinity (*P <0.05,* Fig. 1A) and endophytic fungi (*P <0.05,* Fig. 1A) significantly affected the emergence rate of barley seedlings. Moreover, emergence rate of salt-stressed barley was increased by fungal endophytes (*P<0.05,* Fig. 1A). Barley plants had a high emergence rate (100%) in both colonized and uncolonized plants under non-saline (2.5dS/m) and moderate saline conditions (8dS/m). However, high salinity (14dS/m) reduced barley emergence rate for non-colonized barley (60%), *F. equiseti*, and *F. graminearum* colonized barley (36.7 and 53.3%, respectively), while *E. phragmospora* increased emergence rate to 66.7%.

Salinity significantly decreased root length and all tested endophytes had significant effects on root length (*P*<0.05, Fig. 1B). Root growth showed significant differences between non-inoculated barley plants and the inoculated ones (*P*<0.05, Fig. 1B). Salinity significantly decreased both root (*P*<0.05, Fig. 1C) and shoot (*P*<0.05, Fig. 1D) dry weights. The tested endophytic fungi had significant impact on root (*P>0.05*, Fig. 1C) and shoot dry weight (*P*<0.05, Fig. 1D), indicating that inoculations of barley by fungal endophytes increased root and shoot biomass produced by salt-stressed plants (*P*<0.05*,* Fig. 1C and D).

Overall, salinity reduced barley emergence, growth (root length and leaf area) and biomass production. The results are consistent with other studies which have shown that barley growth is negatively affected by increasing salinity levels (Mallek-Maalej *et al*., 2004; El Goumi *et al*., 2014). Moreover, Albacete *et al*. (2008) reported that salinity affects the cytokinins / auxins hormonal balance disturbing growth and biomass production. On the other hand, the current study supports the concept that beneficial endophytic association enhances plants growth and metabolism under salt stress. The tested endophytes promoted emergence rate, root length and biomass production (shoot and root dry weight) of barley and also helped the plants to tolerate elevated salt stress as compared to control plants.

Endophytic fungi have shown to control some physiological and biochemical processes under stressful conditions (Dardanelli *et al*., 2009). Hamayun *et al*. (2010) revealed that endophytic could reduce the negative effects of salinity and progress plant germination, emergence, growth, and biomass production, by synthesizing phytohormones (gibberellins: GA1, GA4, GA8 and GA9, indole-acetic acid: IAA and abscissic acid: ABA). Also, Yurieva *et al*. (2018) reported that *Penicillium funiculosum* stimulated soybean growth and improved the dry root biomass. Furthermore, Ahmad *et al*. (2015) showed that the increase in plant growth and biomass production after *Trichoderma spp*. inoculation may be due to its ability to produce gibberellins and cytokines, which cannot only promote growth but also pro-

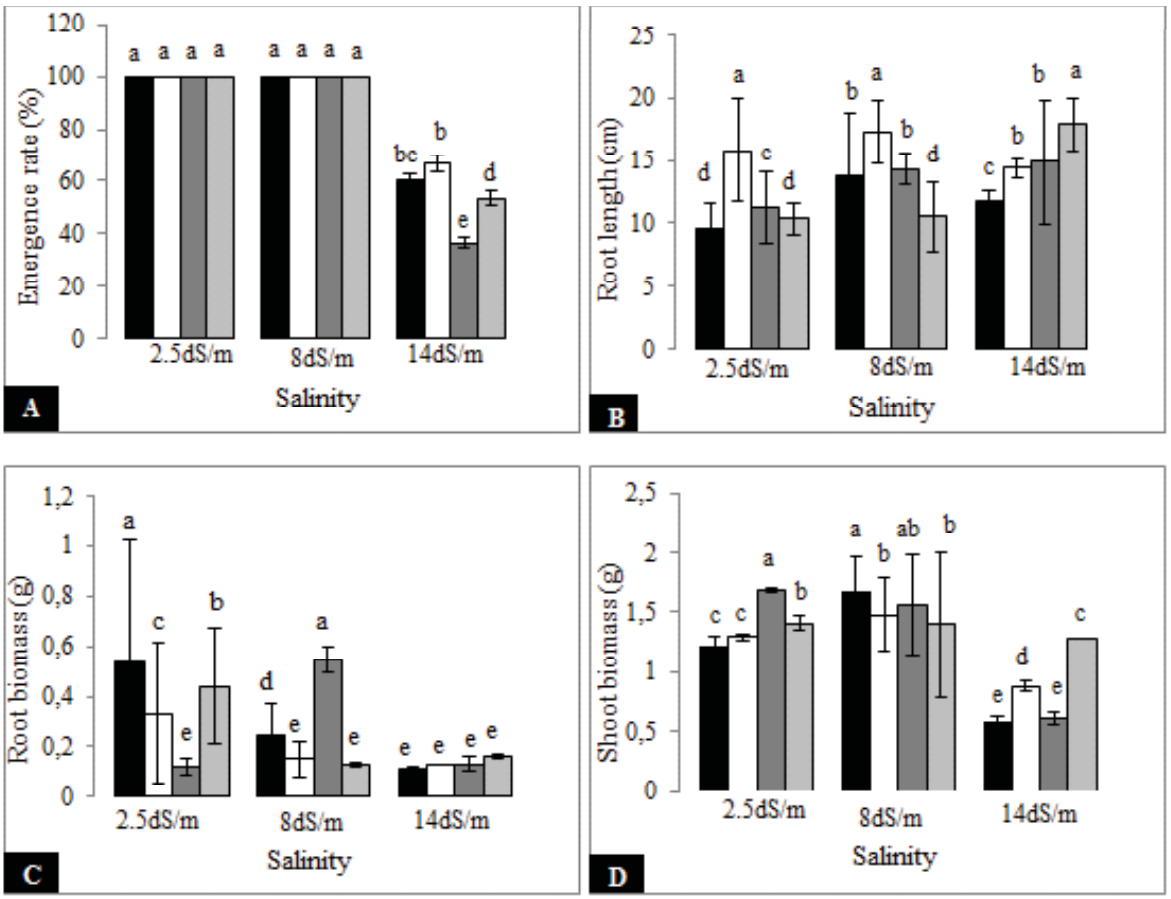

Figure 1. Emergence rate (A), Root length (B), Root biomass (C) and Shoot biomass (D) of barley non-treated (C0) or treated with *Embellisia phragmospora* (EP), *Fusarium equiseti* (FE), and *Fusarium graminearum* (FG) under non-saline and saline conditions. Means followed by the same letter are not significantly different ($P<0.05$).

C0 EP FE FG

vide plants salt stress tolerance.

The relative water content (RWC) was measured in order to indicate cellular volume and response level of plant to environmental stresses (Tátrai *et al*., 2016). Kara and Brinis (2012) indicated that keeping high RWC under saline conditions is a main strategy for maintaining optimal growth of plants under salinity. In the current study, the RWC decreased as salt concentration rose, while RWC values of salt-stressed plants were significantly increased by the tested endophytes under high saline conditions compared to the control plants ($P<0.05$, Fig. 2A). For instance, RWC of inoculated barley plants under non-saline stress increased in *F. equiseti* (81.3% RWC) associated barley as compared to *E. phragmospora* (68.3%) and *F. graminearum* (66.8% RWC) associated barley and control plants (68.12% RWC). While, under moderate saline conditions the RWC decreased with colonization of the tested endophytes and increased in control plants (82% RWC). However, *F. equiseti*-associated barley recorded the highest RWC under high salt stress (78.6% RWC) followed by *E. phragmospora*-inoculated barley (58.4% RWC). The present finding suggests that the effect of fungi on the RWC is greater, which is in close agreement with findings of Hashem *et al*. (2014) and Zhang *et al*. (2016). Siddiqui *et al*. (2014) showed that salinity significantly decreased RWC of rice crops; however, a significant increase was revealed due to *Trichoderma spp.* inoculation.

Mineral nutrition data indicated that the sodium (Na^+) leaf content increased in salt stress treatments as compared to the con-

trol. Regarding the effect of endophytes on Na^+ concentration under salt-stress conditions, plant colonized with endophytes decreased Na^+ concentration compared with the endophyte-free control plants ($P<0.05$, Fig. 2B). Potassium (K^+) leaf content decreased in salt-stressed barley compared to control plants; however, endophytic fungi increased K^+ concentration compared to non-inoculated plants ($P<0.05$, Fig. 2C). Bouzid (2010) reported that sodium absorption by plants is caused due to increases in NaCl and Na_2SO_4 concentrations in the culture medium.. Haouala *et al*. (2007) showed that potassium contents of ryegrass leaves and roots were reduced at salinity exceeding of 50 mM NaCl.

The endophytes *E. phragmospora* and *F. graminearum* decreased Na^+ content under moderate and high salinity as compared to non-inoculated barley. Inoculation of *E. phragmospora* increased K^+ content compared to the non-inoculated barley under moderate salinity while in severe salinity *E. phragmospora*, *F. equiseti* and *F. graminearum*-colonized plants had higher K^+ content as compared to non-associated ones. Li *et al*. (2017) reported that endophytic fungi can protect plants against the toxic effects of ions by attenuating Na^+ absorption in saline conditions and conserving high K^+ assimilation. Moreover, endophytic fungi can be a source of minerals (Shankar *et al*., 2008). Ghorbani *et al*. (2019) indicated that *Piriformospora indica* improved K^+/Na^+ homoeostasis.

Regarding the sugar content results, salt treatment significantly decreased sugar leaves content in barley ($P<0.05$, Fig. 3B). When barley was grown under salt-stressed greenhouse conditions, none of fungal endosymbionts tested had a significant impact on soluble sugar levels ($P>0.05$; Fig. 3B); however, plants subjected to salt stress and colonized by endophytic fungi had greater proline content than uninoculated plants ($P<0.05$, Fig. 3A). Widodo *et al*. (2009) revealed that environmental changes affected plants metabolic homeostasis. Hu *et al*. (2014) exhibited that plants exposed to salt

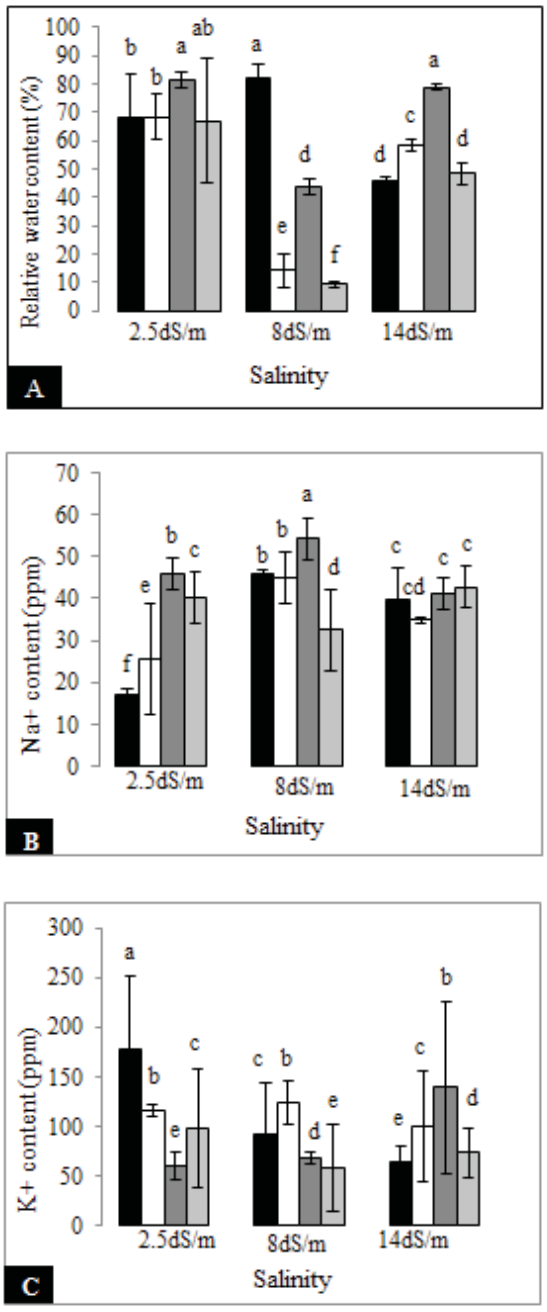

Figure 2. Relative water (A), Na+ (B) and K+ (C) contents of barley non-treated (C0) or treated with *Embellisia phragmospora* (EP), *Fusarium equiseti* (FE), and *Fusarium graminearum* (FG) under non-saline and saline conditions. Means followed by the same letter are not significantly different ($P<0.05$).

stress stimulate a range of metabolic responses, by producing several metabolites, such as proline and sugars.

The sugar content in barley decreased with increasing salinity. The current results

are in agreement with those of Liu and Staden (2001), showing that salt-tolerant soybeans are characterized by a decrease in sucrose accumulation under saline conditions. Nevertheless, *F. graminearum* increased sugar content compared to non-inoculated barley and the other endophytes. The study of Sampangi-Ramaiah *et al*. (2020) demonstrates that a salt tolerant endophyte, *Fusarium sp*., from the salt-adapted Pokkali rice, can be successfully transferred to the cultivated salt-sensitive rice variety IR-64 to confer salt tolerance. Li *et al*. (2017) suggested that the high accumulation of soluble sugars in *Aspergillus aculeatus*-associated perennial ryegrass could contribute to plant protective mechanisms under salt stress by adjusting the osmotic balance.

Our data showed that proline levels increased with increasing salt concentration. Similar results have been shown in barley (Zerrad *et al*., 2008), durum wheat (Chorfi, 2009) and rice (Joseph *et al*., 2015). Proline is one of the osmolytes accumulated by plants under stressful conditions; its accumulation has a role in protecting the cell membrane and participating in osmotic adjustment (Verbruggen and Hermans 2008). Inoculated barley had higher proline content compared to non-inoculated plants. These results agree with those of Dardanelli *et al*. (2009), reporting an increase in proline content in plants colonized by endophytes under saline stress. High proline contents have been reported in *Aspergillus aculeatus*-colonized perennial ryegrass under salt stress and therefore the endophyte was hypothesized to increase the ability of the plants to tolerate salt stress (Li *et al*., 2017).

Results showed a significant increase in chlorophyll a ($P<0.05$, Fig. 4A), chlorophyll b ($P<0.05$, Fig. 4B), chlorophyll total ($P<0.05$, Fig. 4C) and carotenoids ($P<0.05$, Fig. 4D) biosynthesis with increase in salt concentration. However, the tested endophytic fungi had no significant impact on photosynthetic pigments biosynthesis since the pigments (chlorophyll a, b, total and carotenoids) in colonized and salt-stressed barley did not differ from that of control plants ($P>0.05$; Fig. 4).

Rahneshan *et al*. (2018) revealed that salt tolerant plants have increased or unchanged levels of chlorophyll under saline conditions, while chlorophyll levels decrease in salt sensitive plants. Results obtained by Zraibi *et al*. (2012) have shown that carotenoids are an effective antioxidant that protects and stabilizes photochemical processes of photosynthesis under stressful conditions. According to Ghorbani *et al*. (2018), inoculation of tomato plants with *Piriformospora indica* can promote plant growth via improving gas exchange, water potential, chloro-

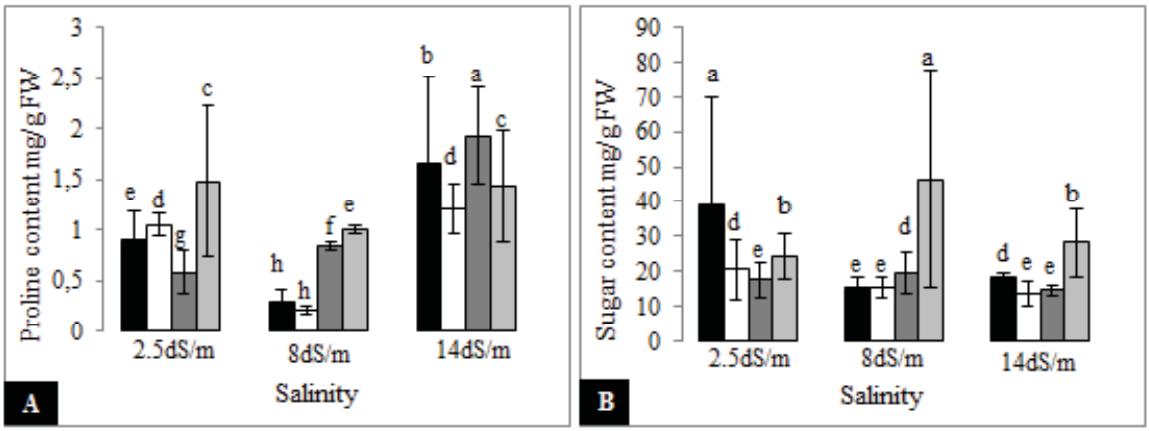

Figure 3. Proline (A) and Sugar (B) contents of barley non-treated (C0) or treated with *Embellisia phragmospora* (EP), *Fusarium equiseti* (FE), and *Fusarium graminearum* (FG) under non-saline and saline conditions. Means followed by the same letter are not significantly different ($P<0.05$).

C0 EP FE FG

phyll content and chlorophyll fluorescence parameters.

Our results on inoculation of endophytic fungi on barley in saline environment are similar to those by Ban *et al*. (2017) showing that inoculation of the endophytic fungus *Gaeumannomyces Cylindrosporus* had no significant effect on photosynthetic pigments in maize plants under stressful conditions. However, Jogawat *et al*. (2013) indicated that *Piriformospora indica* enhanced barley and rice salt stress tolerance by increasing the activity of photosynthetic pigments in colonized plants. Xie *et al*. (2014) suggested that *Aspergillus aculeatus* can colonize plants and improve photosynthesis and chlorophyll content and facilitate plant growth under stress.

The present study has revealed that endophytic fungi appear to confer salt tolerance of barley as evident by changes in physiological and biochemical indexes, involving several mechanisms. As the mechanisms by which endophytic fungi interact with host plants and increase plant growth and yield under salt stress are incompletely understood, future *in vitro* and *in planta* research into the cellular, epigenetic and molecular mechanisms is merited. The capacity of endophytic fungi to increase barley tolerance under salinity stress and to improve growth could be applicable to achieve a sustainable agriculture.

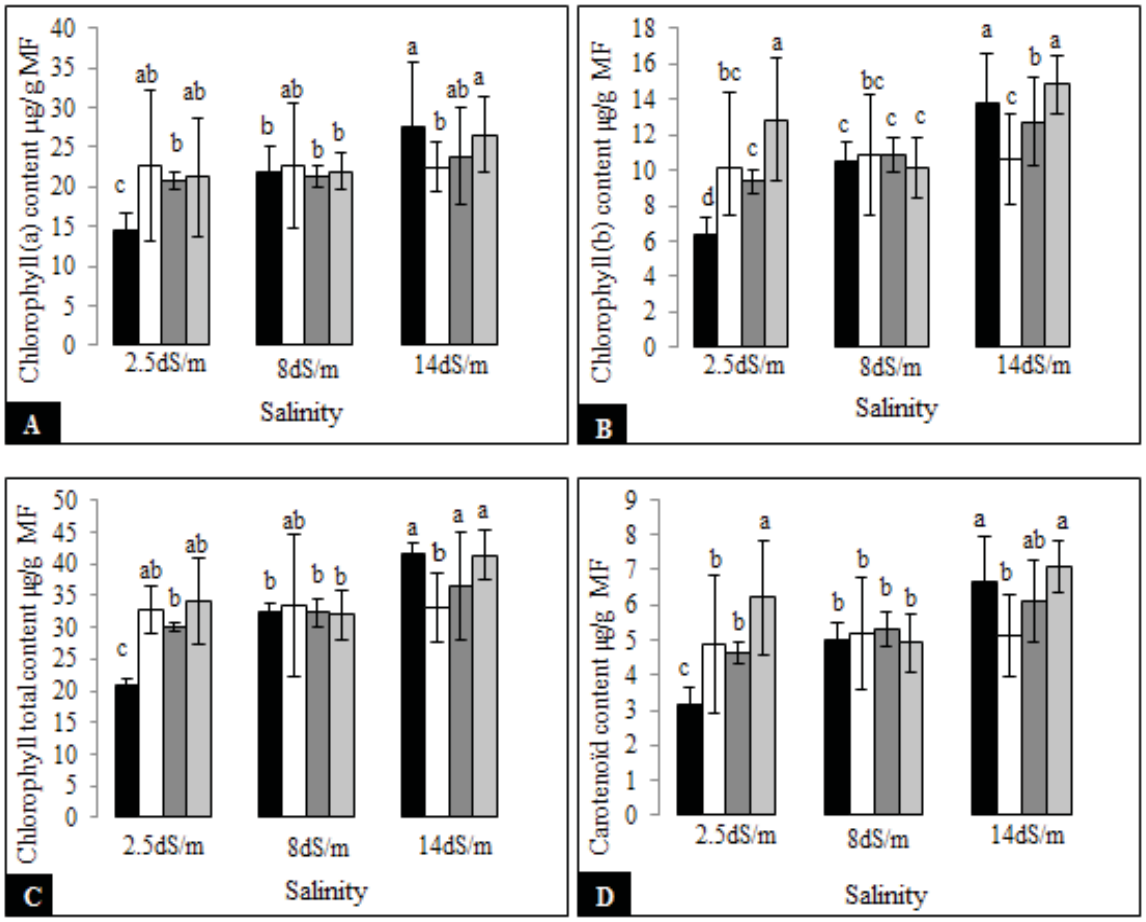

Figure 4. Chlorophyll a (A), Chlorophyll b (B), Chlorophyll total (C) and Carotenoid (D) contents of barley non-treated (C0) or treated with *Embellisia phragmospora* (EP), *Fusarium equiseti* (FE), and *Fusarium graminearu*m (FG) under non-saline and saline conditions. Means followed by the same letter are not significantly different ($P<0.05$).

C0 EP FE FG

Literature cited

Aghilia, F., Jansab, J., Khoshgoftarmanesh, A.H., Afyunic, M., Schulind, R., Frossarda, E. and Gampera, H.A. 2014. Wheat plants invest more in mycorrhizae and receive more benefits from them under adverse than favorable soil conditions. *Applied Soil Ecology*, 84: 93-111.

Ahmad, P., Abeer, H., Elsayed, F.A.A., Alqarawi, A.A., Riffat, J., Dilfuza, E. and Salih, G. 2015. Role of *Trichoderma harzianum* in mitigating NaCl stress in Indian mustard (*Brassica juncea* L.) through antioxidative defense system. *Frontiers in Plant Science,* 6: 868.

Akagi, A., Jiang, C.J. and Takatsuji, H. 2015. *Magnaporthe oryzae* Inoculation of Rice Seedlings by Spraying with a Spore Suspension. *Bio-Protocol,* 11: 1-5.

Albacete, A., Ghanem, M.E., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Martínez, V., Lutts, S., Dodd, I.C. and Pérez-Alfocea F. 2008. Hormonal changes in relation to biomass partitioning and shoot growth. Impairment in salinized tomato (*Solanum lycopersicum* L.) plants. *Journal of Experimental Botany* 59 (15): 4119-4131.

Baenziger, M., Setimela, P.S., Hodson, D. and Vivek, B. 2006. Breeding for improved abiotic stress tolerance in maize adapted to southern Africa. *Agricultural Water Management,* 80: 212-224.

Ban, Y., XU, Z., Yang, Y., Zhang, H., Chen, H., and Tang, M. 2017. Effect of dark septate endophytic fungus *Gaeumannomyces cylindrosporus* on plant growth, photosynthesis and Pb tolerance of maize (*Zea mays* L.). *Pedosphere,* 27: 283–292.

Bouzid, N. 2010. Étude de la résistance d'*Atriplex halimus* subsp. schweinfurthii aux sels solubles. *Acta Botanica Gallica*, 157 (4): 787-791.

Chapman, H.D. and Pratt, P.F. 1961. Methods of Analysis for Soils, Plants and Water. Univ. California, Public Division of Agricultural Sciences, Berkeley, CA, USA. 150-179.

Chorfi, A. 2009. Contribution à l'étude de la résistance à la salinité chez une variété de blé dur algérien (*Triticum durum* Desf.) Var. mohamed ben bachir. *Sciences and Technologie,* 29: 41-44.

Dardanelli, M.S., Manyani, H., Gonzalez-Barroso, S., Rodriguez-Carvajal, M.A., Gil-Serrano A.M., Espuny, M.R., López-Baena, F.J., Bellogín, R.A., Megías, M. and Ollero, F.J. 2009. Effect of the presence of the plant growth promoting rhizobacterium (PGPR) *Chryseobacterium balustinum* Aur9 and salt stress in the pattern of flavonoids exuded by soybean roots. *Plant Soil,* 328: 483-493.

El Goumi, Y., Fakiri, M., Lamsaouri, O. and Benchekroun, M. 2014. Salt stress effect on seed germination and some physiological traits in three Moroccan barley (*Hordeum vulgare* L.) cultivars. *Journal of Materials and Environmental Science*, 5: 625-632.

Ghorbani, A., Omran, V.O.G., Razavi, S.M., Pirdashti, H. and Ranjbar, M. 2018. *Piriformospora indica* inoculation alleviates the adverse effect of NaCl stress on growth, gas exchange and chlorophyll fluorescence in tomato (*Solanum lycopersicum* L.). *Plant Biology,* 20: 729-736.

Ghorbani, A., Omran, V.O.G., Razavi, S.M., Pirdashti, H. and Ranjbar, M. 2019. *Piriformospora indica* confers salinity tolerance on tomato (*Lycopersicon esculentum* Mill.) through amelioration of nutrient accumulation, K^+/Na^+ homeostasis and water status. *Plant Cell Reports*. Springer-Verlag GmbH Germany, part of Springer Nature 2019. doi.org/10.1007/s00299-019-02434-w.

Gill, S.S., Gill, R., Trivedi, D.K., Anjum, N.A., Sharma, K.K., Ansari, M.W., Ansari, A.A., Johri, A.K., Prasad, R., Pereira, E., Varma, A. and Tuteja, N. 2016. *Piriformospora indica*: potential and significance in plant stress tolerance. *Frontiers in Microbiology,* 7: 332.

Gupta, B. and Huang, B. 2014. Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *International Journal of Genomics*, 2014:701596. https ://doi.org/10.1155/2014/70159 6.

Hamayun, M., Khan, S.A., Khan, A.L., Tang, D.S., Hussain, J., Ahmad, B., Anwar, Y. and Lee, I.J. 2010. Growth promotion of cucumber by pure cultures of gibberellin-producing *Phoma sp*. GAH7. *World Journal of Microbiology and Biotechnology,* 26: 889-894.

Haouala, F., Ferjani, H. and El Hadj, S. 2007. Effet de la salinité sur la répartition des cations (Na+, K+ et Ca+2) et du chlore (Cl-) dans les parties aériennes et les racines de ray-grass anglais et du chiendent. *Biotechnology, Agronomy, Society and Environment*, 11 (3): 235-244.

Hasan, H.A.H. 2002. Gibberellin and auxin production by plant root-fungi and their biosynthesis under salinity-calcium interaction. *Rostlinná výroba*., 48: 101-106.

Hashem, A., Abd-allah, E.F., Alqarawi, A.A., Al-Huqail, A.A. and Egamberdieva, D. 2014. Alleviation of abiotic salt stress in *Ochradenus baccatus* (Del.) by *Trichoderma hamatum* (Bonord.) Bainier. *Journal of Plant Interactions,* 9: 857-868.

Hu, L., Chen, L., Liu, L., Lou, Y., Amombo, E. and Fu, J. 2014. Metabolic acclimation of source and sink tissues to salinity stress in bermudagrass (*Cynodon dactylon*). *Plant Physiology,* 155: 166-179.

I.N.S.I.D. 2008. Etat d'avancement du projet (ALG/5/022) de coopération technique avec l'AIEA. Les techniques nucléaires pour l'utilisation durable des terres salines et des eaux saumâtres pour la production agricole. 48p.

Jogawat, A., Saha, S., Bakshi, M., Dayaman, V., Kumar, M., Dua, M., Varma, A., Oelmüller, R., Tuteja, N. and Johri, A.K. 2013. *Piriformospora indica* rescues growth diminution of rice seedlings during high salt stress. *Plant Signaling and Behaviour*, 8: e26891.

Joseph, E.A., Radhakrishnan, V.V. and Mohanan, K.V. 2015. A Study on the Accumulation of Proline- An Osmoprotectant Amino Acid under Salt Stress in Some Native Rice Cultivars of North Kerala. *India Universal Journal of Agricultural Research,* 3 (1): 15-22.

Kara, K. and Brinis, L. 2012. Réponse Physiologique au Stress Hydrique de Variétés de Blé Tendre (*Triticum Aestivum* L.) Cultivées en Algérie. *European Journal of Scientific Research,* 81: 524-532.

Kouadria, R., Bouzouina, M., Azzouz, R. and Lotmani, B. 2018. Salinity stress resistance of durum wheat (*Triticum durum*) enhanced by fungi. *International Journal of Biosciences,* 12: 70-77.

Kouadria, R., Bouzouina, M. and Lotmani, B. 2019. Endophytic fungi contribution in adverse factors tolerance of cultivated species: isolation of endophytic fungi and study of their contribution to salinity or pollutants tolerance (in French). PhD dissertation. 159p.

Kouadria, R., Bouzouina, M. and Lotmani, B. 2020. Induction of growth and osmoregulation in salt stressed barley by the endophytic fungus *Chaetomium coarctatum*. *Tunisian Journal of Plant Protection,* 15: 19-27.

Leitão, A.L. and Enguita, F.J. 2016. Gibberellins in *Penicillium* strains: Challenges for endophyte-planthost interactions under salinity stress. *Microbiological Research,* 183: 8-18.

Li, X., Han, S., Wang, G., Liu, X., Amombo, E., Xie, Y. and Fu, J. 2017. The Fungus *Aspergillus aculeatus* Enhances Salt-Stress Tolerance, Metabolite Accumulation, and Improves Forage Quality in Perennial Rye grass. *Frontiers in Microbiol*ogy, 8: 1664.

Li, F.S., Kang, S.Z. and Zhang, J. 2004. Interactive effects of elevated CO2, nitrogen and drought on leaf area, stomatal conductance, and evapotranspiration of wheat. *Agricultural Water Management,* 67: 221-233.

Liu, T. and Staden, J.V. 2001. Partitioning of carbohydrates in salt sensitive and salt-tolerant soybean callus cultures under salinity stress and its subsequent relief. *Journal of Plant Growth Regulation,* 33: 13.

Majumder, A.L., Sengupta, S. and Goswani, L. 2010. Osmolyte regulation in abiotic stress. Chap dans Abiotic stress adaptation in plants: Physiological, molecular and genomic foundation. Sous la direction de A. Pareek, S.K. Sopory, H.J. Bohmert et Govindjee. p. 349-370.

Mallek-Maalej, E., Boulasnem, F. and Ben Salem, M. 2004. Effet de la salinité sur la germination de graines de céréales cultivées en Tunisie. *Cahiers Agriculture,* 12: 6-153.

Munns, R. and Tester, M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology,* 59: 651-81.

Munns, R., James, R. A., and Läuchli, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany,* 57: 1025-1043.

Rahneshan, Z., Nasibi, F. and Ahmadi Moghadam, A. 2018. Effects of salinity stress on some growth, physiological, biochemical parameters and nutrients in two pistachio (*Pistacia vera* L.) rootstocks. *Journal of Plant Interactions,* 13: 73-82.

Robert-Seilaniantz, A, Navarro, L, Bari, R. and Jones, JD. 2007. Pathological hormone imbalances. *Current Opinion in Plant Biology,* 10:372-9.0.

Rodriguez, R., White, J., Arnold, A. and Redman R. 2009. Fungal endophytes: diversity and functional roles. *New Phytologist,* 182: 314-330.

Ruiz-Lozano, J.M., Porcel, R., Azcón, R. and Aroca, R. 2012. Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies. *Journal of Experimental Botany,* 63: 4033-4044.

Sampangi-Ramaiah, M.H., Jagadheesh, Dey, P., Jambagi, S., Vasantha-Kumari, M.M., Oelmüller, R., Nataraja, K.N., Venkataramana-Ravishankar, K., Ravikanth, G. and Uma-Shaanker, R. 2020. An endophyte from salt-adapted Pokkali rice confers salt-tolerance to a salt-sensitive rice variety and targets a unique pattern of genes in its new host. *Science Reports,* 10: 32-37.

Schields R. and Burnett W. 1960. Determination of protein- bound carbohydrate in serum by a modified anthrone method. *Analytical Chemistry,* 32: 885- 886. https://doi.org/10.1021/ac60163a053.

Scippa, G., Di Michel, M., Onelli, E., Patrignani, G., Chiatante, D. and Bray, E. 2004. The histone-like protein H1-S and the response of tomato leaves to water deficit. *Journal of Experimental Botany,* 55: 99-109.

Shankar Naik, B., Shashikala, J. and Krishnamurthy, Y.L. 2008. Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India. *Fungal Ecology,* 1: 89-93.

Siddiqui, Z.S., Cho, J.I., Hanpark, S., Kwon, T.R., Ahn, B.O., Lee, G.S., Jeong, M.J., Whankim, K., Konlee, S. and Chulpark, S. 2014. Phenotyping of rice in salt stress environment using high-throughput infrared imaging. *Acta Botanica Croatica,* 73: 149-158.

Tátrai, Z.A., Sanoubar, R., Pluhár, Z., Mancarella, S., Orsini, F. and Gianquinto, G. 2016. Morphological and physiological plant responses to drought stress in *Thymus citriodoru*s. *International Journal of Agronomy,* 2: 1-8.

Troll, W., and Lindsley, J. 1955. A photometric method for determination of proline. *Journal of Biological Chemistry,* 215: 655-660.

Verbruggen, N. and Hermans, C. 2008. Proline accumulation in plants: a review. *Amino Acids* 35 (4): 753-759.

Wang, W.X., Vinocur, B. and Altman, A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta,* 218: 1-14.

Wei, Z. and Jousset, A. 2017. Plant breeding goes microbial. Trends Plant Sci 22: 555-558.

Widodo, J.H., Newbigin, E., Tester, M., Bacic, A. and Roessner, U. 2009. Metabolic responses to salt stress of barley (Hordeum vulgare L.) cultivars, sahara and clipper, which differ in salinity tolerance. *Journal of Experimental Botany*, 60: 4089-4103.

Yang, Y.L., Guo, J.K., Zhang, F., Zhaob, L.Q. and Zhang, L.X. 2004. NaCl induced changes of the H^+-ATPase in root plasma membrane of two wheat cultivars. *Plant Science*, 166: 913-918.

Yun, P., Xu, L., Wang, S.S., Shabala, L., Shabala, S. and Zhang, W.Y. 2018. *Piriformospora indica* improves salinity stress tolerance in *Zea mays* L. plants by regulating Na^+ and K^+ loading in root and allocating K+ in shoot. *Journal of Plant Growth Regulation,* 86 (2): 323-331.

Yurieva, O.M., Syrchin, S.O., Nakonechna, L.T. and Kurchenko, I.M. 2018. Influence of endophytic and saprotrophic *Penicillium* funiculosum strains on resistance of *Glycine max* L. under salt stress. *Біологія, біотехнологія, екологія* (*Biology, Biotechnology, Ecology*) 2 (72): 579-262.

Zerrad, W., Hillali, S., Mataoui, B., El Antri, S. and Hmyene, A. 2008. Comparative study of the biochemical and molecular mechanisms of resistance to water stress of two varieties of durum wheat. *Lebanese Science Journal*, 9: 27-36.

Zhang, S., Gan, Y. and Xu, B. 2016. Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 enhances tolerance of wheat to salt stress through improvement of antioxidative defense system and gene expression. *Frontiers in Plant Science,* 7: 1405.

Zraibi, L., Nabloussi, A., Merimi, J., El Amrani, A., Kajeiou, M., Khalid, A. and Serghini Caid, H. 2012. Effet du stress salin sur des paramètres physiologiques et agronomiques de différentes variétés de carthame (*Carthamus tinctorius* L.). AFRIMED AJ - *Al Awamia,* 125: 15-40.

Received: 15 April 2020; Accepted: 6 December 2022

Διερεύνηση του ρόλου των ενδοφυτικών μυκήτων στην ανοχή του κριθαριού στην αλατότητα

R. Kouadria, M. Bouzouina, B. Lotmani και S. Soualem

Περίληψη Η αλατότητα του εδάφους είναι ένα γεωργικό και οικο-περιβαλλοντικό πρόβλημα σε παγκόσμιο επίπεδο, που προκαλεί μείωση της παραγωγής στις καλλιέργειες. Έχει αποδειχθεί ότι οι ενδοφυτικοί μύκητες βελτιώνουν την ανοχή των φυτών σε συνθήκες καταπόνησης. Σκοπός της εργασίας είναι να διερευνήσει σε συνθήκες θερμοκηπίου την αποτελεσματικότητα των ενδοφυτικών μυκήτων *Embellisia phragmospora, Fusarium equiseti* και *F. graminearum* στη βελτίωση της ανοχής φυτών κριθαριού που αναπτύσσονται σε γλάστρες σε διαφορετικά επίπεδα αλατότητας του εδάφους (2,5, 8 και 14dS/m) με βάση την ανάπτυξη, τη σχετική περιεκτικότητα σε νερό και μεταλλικά στοιχεία, τη βιοσύνθεση φωτοσυνθετικών χρωστικών, και τα επίπεδα προλίνης και σακχάρων των φυτών. Τα αποτελέσματα έδειξαν ότι, σε αλατότητα εδάφους 14dS/m, ο μύκητας *E. phragmospora* αύξησε την έκπτυξη των φυταρίων κριθαριού στο 66,7% σε σχέση με το 60% έκπτυξη που καταγράφηκε στο κριθάρι χωρίς ενδόφυτα. Σε αλατούχο έδαφος, οι ενδοφυτικοί μύκητες αύξησαν το μήκος της ρίζας του κριθαριού και το ξηρό βάρος του στελέχους και της ρίζας, μείωσαν τη συσσώρευση Na+ και βελτίωσαν την πρόσληψη K+. Επίσης, σε συνθήκες υψηλής αλατότητας, φυτά με τα ενδόφυτα *F. equiseti* και *F. graminearum* είχαν αυξημένη περιεκτικότητα σε προλίνη ενώ φυτά με *F. graminearum* εμφάνισαν τη μεγαλύτερη περιεκτικότητα σε σάκχαρα. Τα αποτελέσματα δείχνουν τη σημασία των ενδοφυτικών μυκήτων στη βελτίωση της ανοχής του κριθαριού στην καταπόνηση λόγω αλατότητας του εδάφους ως ένα εν δυνάμει ισχυρό εργαλείο με σημαντικά οφέλη στις καλλιέργειες στο πλαίσιο της βιώσιμης γεωργίας.

sciendo

Hellenic Plant Protection Journal **16:** 23-25, 2023
DOI 10.2478/hppj-2023-0003

SHORT COMMUNICATION

First record of the scale insect *Stotzia ephedrae* in Greece

G.J. Stathas[1*], E.D. Kartsonas[2] and A.I. Darras[2]

Summary The scale insect *Stotzia ephedrae* (Newstead) (Hemiptera: Coccomorpha: Coccidae) was recorded for the first time in Greece on 20 April 2021. It was found on *Ephedra foeminea* Forssk. (Ephedraceae: Gnetales) in Athens. From preliminary studies it was found that *S. ephedrae* is an oviparous, biparental species completing one generation per year.

Additional keywords: *Ephedra foeminea,* Greece, *Stotzia ephedrae*

Stotzia ephedrae (Newstead) (Hemiptera: Coccomorpha: Coccidae) was recorded for the first time in Greece on *Ephedra foeminea* Forssk (Ephedraceae: Gnetales) at Lecabettus Hill (37º58´ N, 23º44´ E, altitude: 270m) in Athens, on 20 April 2021. It was found to be settled on *E. foeminea* shoots as ovipositing female adult with a waxy ovisac (Figs. 1, 2). The confirmation of the species *S. ephedrae* was made by Professor Giuseppina Pellizzari, Dipartimento di Agronomia, Animali, Alimenti, University of Padua, Italy. Vouchers of permanent slides of the scale insect (two slides with one preoviposting female adult on each slide, collected on 26-3-2022, and one slide with two preoviposting female adults, collected on 3-4-2022) are kept in the Laboratory of Agricultural Entomology and Zoology, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese.

The genus *Stotzia* Marchal 1906 (Hemiptera: Coccomorpha: Coccidae) includes the species *S. chrysophyllae, S. ephedrae, S. fuscata* and *S. maxima* (García Morales *et al*., 2016). *Stotzia ephedrae* has been recorded in Algeria (MacGillivray, 1921), Azerbaijan (Ben-Dov, 1993), Egypt (García Morales *et al.,* 2016), France (Foldi and Germain, 2018), Georgia (García Morales *et al.,* 2016),

Figure 1. *Ephedra foeminea* infested by *Stotzia ephedrae.*

Figure 2. *Stotzia ephedrae* ovipositing female with ovisac.

[1] Laboratory of Agricultural Entomology and Zoology, Department of Agriculture, School of Agriculture and Food, University of Peloponnese, GR-241 00 Kalamata, Greece.
[2] Laboratory of Floriculture and Landscape Architecture, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese, GR-241 00 Kalamata, Greece.
* Corresponding author: g.stathas@uop.gr

Iran (Kozár *et al.,* 1996; Fallahzadeh and Japoshvili, 2017), Israel (Bodenheimer, 1935), Italy (Pellizzari, 2003), Morocco (García Morales *et al.,* 2016), Sardinia (Pellizzari, 2003) and Spain (Ben-Dov, 1993).

The host plants of *S. ephedrae*, belong to six plant genera and five Families: *Bupleurum* (Apiaceae), *Asparagus* (Asparagaceae), *Ephedra* (Ephedraceae), *Coronila* (Fabaceae), *Genista* (Fabaceae) and *Tamarix* (Tamaricaceae).

World-wide, 25 scale insect species from four Families have been recorded on *Ephedra* species: four species of Coccidae, 15 of Diaspididae, five of Eriococcidae and one of Pseudococcidae (García Morales *et al.,* 2016). The records in Greece are mainly made by Koroneos (1934): *Leucaspis riccae* Targioni Tozztti (Diaspididae) on *E. (vulgaris) distachya* in Athens and on *E. (campylopoda) foeminea* in Lecabettus Hill - Athens and in Ano Lechonia - Magnessia; *Dynaspidiotus (Aspidiotus) ephedrarum* (Lindinger) on *E. (campylopoda) foeminea* in Lecabettus Hill - Athens. The latter species was recorded later on *Ephedra distachya* in Kato Sounio, near Athens (Szita*et al.,* 2017).

Preliminary studies on biology and ecology of *S. ephedrae,* after its first record in Athens, show that the scale is an oviparous biparental species (Fig. 3) completing one generation per year. Also, natural enemies of the scale are recorded, including coccinellid predators and hymenopteran endoparasites (personal communication).

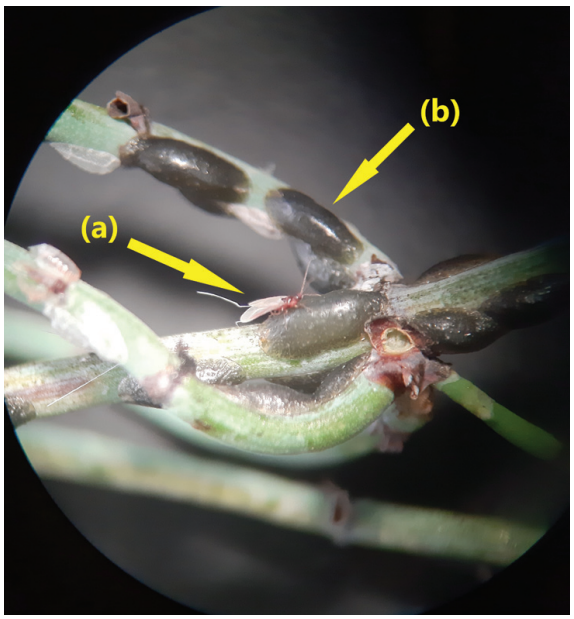

Figure 3. *Stotzia ephedrae* adulds: (a): male, (b): preovipositing female.

The senior author expresses his gratitude to Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti), University of Padua, Italy, for the confirmation of Stotzia ephedrae.

Literature cited

Ben-Dov, Y. 1993. *A systematic catalogue of the soft scale insects of the world (Homoptera: Coccoidea: Coccidae).* Sandhill Crane Press Gainesville, FL, 536 pp.

Bodenheimer, F.S. 1935. Studies on the zoogeography and ecology of palearctic Coccidae I-III. EOS 10: 237-271.

Fallahzadeh, M. and Japoshvili, G.O. 2017. An updated checklist of Iranian Encyrtidae (Hymenoptera, Chalcidoidea). *Zootaxa*, 4344 (1): 001–046.

Foldi, I., and Germain, J.F. 2018. Liste des Cochenilles de France (Hemiptera, Coccomorpha) [Checklist of the scale insects of France (Hemiptera, Coccomorpha)]. *Bulletin de la Societe Entomologique de France*, 123(1): 7-18.

García Morales M, Denno, B.D., Miller, D.R., Miller, G.L., Ben-Dov, Y. and Hardy, N.B. 2016. *ScaleNet: A literature-based model of scale insect biology and systematics*. Retrieved June 23, 2022, from http://scalenet.info.

Kozár, F., Fowjhan, M.A. and Zarrabi, M. 1996. Checklist of Coccoidea and Aleyrodoidea (Homoptera) of Afghanistan and Iran, with additional data to the scale insects of fruit trees in Iran. *Acta Phytopathologica et Entomologica Hungarica,* 31: 61-74.

MacGillivray, A.D. 1921. *The Coccidae.* Scarab Company, Urbana, Illinois, 502pp.

Pellizzari, G., 2003. Hemiptera Coccoidea nuovi o poco noti per l' Italia. [Hemiptera Coccoidea new or little known for the Italian fauna.]. *Bollettino di Zoologia Agraria e di Bachicoltura (Milano)* 35(2): 99-106.

Szita, É., Fetykó, K.G., Benedicty, Z.K., Kozár, F., Partsinevelos, G., Milonas, P. and Kaydan, M.B. 2017. Data on the scale insect (Hemiptera: Coccomorpha) fauna of Greece, with description of two new species. *Zootaxa,* 4329(5): 463-476. doi:10.11646/zootaxa.4329.5.4

Received: 22 July 2022; Accepted: 15 December 2022

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του κοκκοειδούς *Stotzia ephedrae* στην Ελλάδα

Γ.Ι. Σταθάς, Ε.Δ. Κάρτσωνας και Α.Ι. Δάρρας

Περίληψη Η εργασία αποτελεί την πρώτη καταγραφή στην Ελλάδα του κοκκοειδούς εντόμου *Stotzia ephedrae* (Newstead) (Hemiptera: Coccomorpha: Coccidae). Το έντομο βρέθηκε στις 20 Απριλίου 2022 επί του φυτού *Ephedra foeminea* Forssk. (Ephedraceae: Gnetales) στο λόφο του Λυκαβηττού στην Αθήνα. Από τρέχουσες προκαταρκτικές μελέτες διαπιστώθηκε ότι το είδος *S. ephedrae* είναι ωοτόκο, αμφιγονικό και συμπληρώνει μία γενεά το έτος.

Hellenic Plant Protection Journal **16:** 23-25, 2023

sciendo

Hellenic Plant Protection Journal **16:** 26-28, 2023
DOI 10.2478/hppj-2023-0004

SHORT COMMUNICATION

First record of the scale insect *Lineaspis striata* on *Picea glauca* "Conica"

G.J. Stathas[1*], A.I. Darras[2] and E.D. Kartsonas[2]

Summary The scale insect *Lineaspis striata* (Newstead) (Hemiptera: Coccomorpha: Diaspididae) was recorded for the first time infesting a species of the family Pinaceae. Heavily infested ornamental shrubs of *Picea glauca* "Conica" by pre-ovipostiting and oviposting female adults of *L. striata*, were found in March 2018 and by a minor population consisting of pre-ovipositing female adults in February 2021, in Attica, Greece. The scale was settled on the base of the needles of the host plant.

Additional keywords: *Lineaspis striata*, *Picea glauc*a "Conica", scale insect

The scale insect species reported on *Picea glauca* "Conica" belong to the family Diaspididae: *Chionaspis pinifoliae* (Fitch) and *Hemiberlesia ithacae* (Ferris) and Coccidae: *Coccus hesperidum hesperidum* (L.), *Physokermes hemicryphus* (Dalman) and *Physokermes inopinatus* Danzig (García Morales *et al*. 2016). Other arthropod pests of *P. glauca* "Conica" include the curculionids *Otiorhynchus sulcatus* (Fabricius) and *Otiorhynchus ovatus* (Linnaeus) (Coleoptera: Curculionidae) (Fisher, 2006), and the mite *Oligonychus ununguis* Jacobi (Acari: Tetranychidae) which is reported as a serious pest of the plant (Kiełkiewicz *et al.,* 2005)

Lineaspis striata (Newstead) is a scale insect species of the Palearctic region, distributed to Algeria, Armenia, Corsica, Egypt, France, Georgia, Greece, Israel, Jordan, Kazakhstan, Morocco, Sardinia, Spain, Syria and Turkey (Koroneos, 1934; Bodenheimer, 1953; Balachowski, 1954; Avidov and Hapaz, 1969; Pellizzari *et al.,* 2011; Ben-Dov, 2012; Pellizzari *et al.,* 2015; García Morales *et al.,* 2016; Szita *et al.,* 2017; Normark *et al.,* 2019). *Lineaspis striata* has been recorded on host plants species of nine Genera that belong to four plant Families: *Callitris, Cupressus, Juniperus, Platycladus, Tetraclinis, Callitris* and *Thuja* (Cupressaceae), *Iris* (Iridaceae), *Arceuthobium* (Santalaceae) and *Taxus* (Taxaceae) (García Morales *et al*., 2016).

In Greece, *L. striata*has been recorded by Koroneos (1934) under the synonym *Chionaspis striata* Newstead on *Thuja orientalis* in Volos, on *Cupressus sempervirens* in Peloponnese and Pelion, on *Juniperus macrocarpa* in Voula (Attica) and on *Juniperus phoenicea* in Vouliagmeni (Attica). Later it was recorded on *Cupressus* sp. in Dionysos (Attica) (Katsoyannos, 1993) and on *Cupressus sempervirens* and *Cupressus* sp. in Crete (Pellizzari *et al*., 2011).

In March 2018 and in February 2021, *L. striata* was found during the present study on shrubs of *Picea glauca* "Conica" (Pinaceae) in a southern suburb of Athens, Ano Glyfada, Attica (37º53´ N, 23º45´ E, altitude: 105m) (Fig.1). The confirmation of the scale species was made by Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti, University of Padua, Italy). This is the first record of *L. striata* on a plant of the family Pinaceae.

In March 2018 high population of *L. stri-*

[1] Laboratory of Agricultural Entomology and Zoology, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese, GR-241 00 Kalamata, Greece.
[2] Laboratory of Floriculture and Landscape Architecture, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese, GR-241 00 Kalamata, Greece.
* Corresponding author: g.stathas@uop.gr

Figure 1. *Lineaspis striata* on *Picea glauca* "Conica".

ata was recorded consisting mainly by pre-ovipositing female adults and less by ovipositing female adults. In February 2021 it was found in a lower infestation level, consisting by pre-oviposting female adults. Moreover, the scale was recorded to be oviparous and at the end of the hibernating period (February and March) as female adult. The scale was settled on the base of the needles of the host plant. The plants were also heavily infested by mites which were not identified. In both years exit holes of parasitoids were recorded on the female adult scale covers but no alive parasitoid was found to enable identification of the species.

According to studies by Katsoyannos (1993) on the phenology and the natural enemies of *L. striata* in Dionysos (Attica), the scale is oviparous, hibernated as pre-ovipositing female adult and completed three generations per year. The start of oviposition of the hibernated females was recorded in May, in July and in September (1st, 2nd and 3rd generation, respectively). The average fecundity was 50 eggs (Standard Deviation: 12.4) per female. The earlier start of oviposition in Ano Glyfada (in March) compared to Dionysos (in May) could be attributed to the different climatic conditions between the two areas (Ano Glyfada is a warmer area than Dionysos) and to differences of the host plants (families Pinaceae and Cupressaceae).

The main natural enemy of the scale reported by Katsoyannos (1993) was the endoparasitoid *Physcus testaceus* Masi (Hymenoptera: Aphelinidae) parasitizing the female adult of the scale (parasitism rate was recorded up to 44.8%). Individuals of the ectoparasitoid *Aphytis* sp. (Hymenoptera: Aphelinidae) and the predator *Chilocorus bipustulatus* L. (Coleoptera: Coccinellidae) were also observed (Katsoyannos, 1993). Other natural enemies of *L. striata* in the bibliography include the parasitoids *Aphytis mytilaspidis* (Le Baron), *Coccobius testaceus* (Masi) (=*Physcus testaceus*), *Encarsia citrina* (Craw) and *Encarsia lounsburyi* (Berlese and Paoli) (Hymenoptera: Aphelinidae), and the predators *Pharoscymnus setulosus* (Chevrolat) and *Pharoscymnus varius* Ahmad (Coleoptera: Coccinellidae) and *Mitromica africana* (Rolán and Fernandes) (Gastropoda: Costellariidae) (García Morales *et al*., 2016).

The senior author expresses his gratitude to Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti, University of Padua, Italy), for the confirmation of Lineaspis striata.

Literature cited

Avidov, Z. and Harpaz, I. 1969. *Plant Pests of Israel*. Israel University Press, Jerusalem, 549 pp.

Balachowsky, A.S. 1954. *Les cochenilles Paléarctiques de la tribu des Diaspidini*. Memmoires Scientifiques de l'Institut Pasteur Paris. 450 pp.

Ben-Dov, Y. 2012. The scale insects (Hemiptera: Coccoidea) of Israel—checklist, host plants, zoogeographical considerations and annotations on species. *Israel Journal of Entomology*, 41–42: 21–48.

Bodenheimer, F.S. 1953. The coccoidea of Turkey. Vol. 2. Rev. fac. Sc. Istanbul ser B.T. 28 (1): 1-164.

Fisher, J.R. 2006. Fecundity, longevity and establishment of *Otiorhynchus sulcatus* (Fabricius) and *Otiorhynchus ovatus* (Linnaeus) (Coleoptera: Curculionidae) from the pacific north-west of the United States of America on selected host plants. *Agricultural and Forest Entomology,* 8(4), 281-287. doi:10.1111/j.1461-9563.2006.00309.x.

García Morales M, Denno, B.D., Miller, D.R., Miller, G.L., Ben-Dov, Y., and Hardy, N.B. 2016. *ScaleNet: A literature-based model of scale insect biology and systematics*. Database. doi: 10.1093/database/bav118. Retrieved September 29, 2022, from http://scalenet.info.

Katsoyannos, P. 1993. Phenology and natural enemies of *Lineaspis striata* Newstead (Homoptera: Diaspididae) in Attica. *Proceedings of the 5th Pan-Hellenic Congress of Entomology*. 8-10 November, Athens, Greece, 201–205.

Kiełkiewicz, M., Puchalska, E., and Czajkowska, B. 2005. Changes in biochemical composition of needles of ornamental dwarf spruce (*Picea glauca* 'Conica') induced by spruce spider mite (*Oligonychus ununguis* Jacobi, (Acari: Tetranychidae) feeding. *Acta Physiologiae Plantarum,* 27(4), 463-472. doi:10.1007/s11738-005-0051-1.

Koroneos, J. 1934. *Les Coccidae de la Grèce surtout du Pélion (Thessalie).* I. Diaspinae. Athens, 95 pp, 77 tables.

Normark, B.B., Okusu, A., Morse, G.E., Peterson, D.A., Itioka, T., & Schneider, S.A. 2019. Phylogeny and classification of armored scale insects (Hemiptera: Coccomorpha: Diaspididae). *Zootaxa*, 4616(1), 1-98. doi:10.11646/zootaxa.4616.1.1

Pellizzari, G., Porcelli, F., Seljak, G. and Kozár, F. 2011. Some additions to the Scale insect fauna (Hemiptera: Coccoidea) of Crete with a check list of the species known from the island. *Journal of Entomological and Acarological Research*, 43 (3): 291-300.

Pellizzari, G., Chadzidimitriou, E., Milonas, P., Stathas, G.J. & Kozár, F. 2015. Check list and zoogeographic analysis of the scale insect fauna (Hemiptera: Coccomorpha) of Greece. *Zootaxa*, 4012 (1): 057-077.

Szita, E., Fetykó, G., Benedicty, Z.K., Kozár, F., Partsinevelos, G., Milonas, P., Kaydan, M.B. 2017. Data on the scale insect (Hemiptera: Coccomorpha) fauna of Greece, with description of two new species. *Zootaxa*, 4329 (5): 463-476.

Received: 5 October 2022; Accepted: 24 December 2022

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του κοκκοειδούς εντόμου *Lineaspis striata* σε *Picea glauca* "Conica"

Γ.Ι. Σταθάς, Α.Ι. Δάρρας και Ε.Δ. Κάρτσωνας

Περίληψη Το κοκκοειδές έντομο *Lineaspis striata* (Newstead) (Hemilptera: Coccomorpha: Diaspididae) καταγράφηκε για πρώτη φορά να προσβάλλει είδος της Οικογένειας Pinaceae. Βρέθηκαν έντονα προσβεβλημένα φυτά *Picea glauca* "Conica" από ωοτοκούντα και από προωοτοκίας θήλεα ακμαία του *L. striata* τον Μάρτιο του 2018, καθώς και λιγότερο προσβεβλημένα από θήλεα ακμαία προ-ωοτοκίας τον Φεβρουάριο του 2021, στην περιοχή της Άνω Γλυφάδας (Αττική). Το έντομο εγκαθίσταται στη βάση των φύλλων του φυτού ξενιστή του.

Hellenic Plant Protection Journal **16:** 26-28, 2023

sciendo

Hellenic Plant Protection Journal **16:** 29-39, 2023
DOI 10.2478/hppj-2023-0005

Evaluation of the antibacterial activity of essential oil of *Laurus nobilis* against *Pseudomonas syringae* pv. *phaseolicola* and potential biocidal action

S. Mamoucha, A. Prombona and A. Galeou*

Summary With a continuously growing human population on our planet, the chemical burden of our environment is also growing. In addition, the resistance of microorganisms, like bacteria, to widely used chemicals is evident. Therefore, the application of bactericidal products that reduce the risks for development of resistance as well as the environment and human safety is of great benefit. In this work, we have screened the essential oil extracted from plants of *Laurus nobilis* L. (laurel) grown at the base of the Greek mountain Olympus for its antimicrobial activity against two strains of the *Phaseolus vulgaris* pathogen, *Pseudomonas syringae* pv. *phaseolicola* as well as human pathogenic bacteria (biocidal use). Our results, obtained with established methods, like Well diffusion and Disc diffusion assay, reveal that laurel essential oil is a very effective bacteriostatic and bactericidal agent. Importantly, the activity of laurel essential oil as growth inhibitor of the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* is reported for the first time. This opens the field for more extended investigations regarding its use in crop protection. Additionally, the laurel essential oil tested showed significant antibacterial properties against several human pathogenic bacteria, namely *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Additional Keywords: phytopathogenic bacteria, *Pseudomonas syringae* pv. *phaseolicola*, Well- Disc- diffusion assay

Introduction

In modern times there is a continuously growing interest of researchers to explore the bioactive and bioeffective properties of plants for therapeutic or food preservative purposes, as a green alternative or in complement to established synthetic, chemistry-based agents (Elshafie and Camele, 2017). The plant extracts with such biological activity belong to the secondary metabolites (SMs) of plants. Plants which accumulate bioactive SMs are known as "Medicinal plants". The plants that produce also aromatic SMs (volatiles) are known as "Aromatic and Medicinal plants" (AMPs) (Hussein and El-Anssary, 2018).

The Mediterranean Basin is one among the first twenty-five Global Biodiversity Hotspots on our planet with 25.000 native and endemic plant species (Myers *et al*., 2000). Greece, which is located in the Eastern Mediterranean, hosts the largest plant biodiversity of the Basin per unit of area with up to 1520 taxa. This large plant diversity is attributed to the climate, the geographical position and geomorphology of Greece. Interestingly, many of the endemic species are characterized as AMPs (Georghiou and Delipetrou, 2010).

The plant family Lauraceae comprises over 2.500 species. *Laurus nobilis* L. (Lauraceae) is a broadly and wildly growing evergreen AMP which is endemic in the Mediterranean Basin where it grows spontaneously or it is cultivated (Greece, Turkey, Spain, Portugal and Morocco) (Stace, 2010). It is locally known as bay laurel, sweet bay, bay, true laurel, Grecian laurel or laurel tree. Laurel is known from the ancient times for its beneficial medicinal properties (Stefanova *et al*., 2020). Pedanius Dioscurides, the an-

Institute of Biosciences and Applications, Laboratory of Chronobiology, NCSR 'Demokritos', Patr. Gregoriou E & 27 Neapoleos Str., GR-153 41 Agia Paraskevi, Attiki, Greece.
* Corresponding author: galeou@bio.demokritos.gr

cient Greek botanist and pharmacologist, mentions the therapeutic values of this AMP in his book "De Materia Medica". According to this work, crushed leaves can "heal bee and wasp stings and any kind of inflammation" (Dioscurides, 77AC, 2001). As to the Greek mythology, the plant was dedicated to the God Apollo, whose priestess, Pythia, used to chew *L. nobilis* leaves and uttered her famous prophecies (Giesecke, 2014).

The essential oil (EO) of *L. nobilis* has been recognized to possess many interesting medicinal properties such as antimicrobial, antioxidant (Ramos *et al*., 2012), anti-inflammatory (Kupeli *et al*., 2007) and anticarcinogenic (Saab *et al*., 2012) ones. EO extracted from all the parts of this plant is reported to exhibit antimicrobial activity (Bennadja *et al*, 2013). Since laurel is well acknowledged in the field of traditional medicine, the chemical composition of its EO has been studied extensively. The main chemical classes of volatiles in *L. nobilis* EO are oxygenated monoterpenes, monoterpene hydrocarbons, phenylpropanoids, sesquiterpene hydrocarbons and oxygenated sesquiterpenes (Pino *et al*., 1993). The metabolite 1.8-cineole was reported as the major component in the EO of laurel plants from Argentina (Huergo *et al*., 1978), Tunisia (Bouzouita *et al*., 2001), Turkey (Kilic *et al.,* 2005), Croatia–Serbia (Politeo *et al*., 2007), Italy (Flamini *et al*., 2007) and Iran (Moghtader and Farahmand, 2013). The spicy aroma of leaves is attributed to benzene compounds (eugenol, methyl eugenol, and elemicin) present in percentages ranging between 1% and 12% (Pino *et al*., 1993). Additionally, the stressful Mediterranean climate (heat and drought stress) is thought to be one reason for the biosynthesis of a broad spectrum of active metabolites (Mamoucha *et al*., 2018).

The chemical components of EO from *L. nobilis* growing in Greece (mountain Athos) and Georgia, and their antimicrobial activity against 20 human pathogenic microorganisms i.e., *Enterococcus faecalis*, *Staphylococcus aureus* and *Candida albicans* have been formerly studied by the authors (Stefanova *et al*., 2020). Previous work by Bozkurt *et al*. (2020) has provided evidence of inhibitory activity of EO of *L. nobilis* against gall-forming phytopathogenic bacteria (*Rhizobium radiobacter*, *Pseudomonas savastanoi* pv. *savastanoi* and *P. savastanoi* pv. *nerii*). Here, the antibacterial activity of the Greek *L. nobilis* EO is tested on the growth of *Pseudomonas syringae* pv. *phaseolicola*, an important plant pathogen which causes the disease 'halo blight' in *Phaseolus vulgaris*. Halo blight is ascribed globally yield losses of up to 45%, but the phytoprotection against this disease is mainly based on preventive measures. For chemical control, mainly copper preparations are used, which are known to be particularly burdensome for the environment and for the human health (Arnold *et al*., 2011). Moreover, the Greek laurel EO was tested against several human pathogenic bacteria for its potential biocidal activity, namely *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. Our work contributes to the research on development of alternative to chemical compounds for use in crop protection and as biocides.

Material and methods

Plant material

The upper part (young leaves) of *L. nobilis* plants was collected during the period of full flowering, from a cultivated area at Litochoro (300 m altitude), at the base of the eastern side of mountain Olympus (Greece). The plant tissue was collected on sunny days, under an atmospheric temperature of approximately 20°C and was kept under shade, in open wooden basket, for 1 hour until used.

Extraction of essential oils and preparation

The EO extraction process was conducted using the hydrodistillation method, in a modified Clevenger-type apparatus for 3 h with 3 l of H_2O. One thousand grams of leaves were extracted and yielded 9 ml of EO. Fresh leaves were chopped, placed in a

volumetric flask and water was added. The temperature was maintained at approximately 100°C. EO was removed with a Pasteur pipette and stored at refrigeration temperature (4°C) in glass flasks wrapped in aluminum foil.

For the antibacterial screening, pure and diluted EO was used. The dilution was performed in different emulsifying agents (ethanol 70%, 80%, 90% (v/v), DMSO 0,2%, 0,5%, 5%, 20% (v/v) and Tween20 0,5%, 5%, 20% (v/v)). Additionally, a combination of 0,02% Tween and LB broth (PanReac, U.S.A.) was tested.

Preparation of bacterial cultures

Human pathogenic bacteria, commonly used to evaluate antimicrobial activity of EOs, were obtained from the American Type Culture Collection ATCC; the Gram-positive *Bacillus subtilis* (ATCC9372), *Staphylococcus aureus* (ATCC29213), *Micrococcus luteus* (ATCC9341) and the Gram-negative *Escherichia coli* (ATCC25922). The plant pathogenic bacteria, *Pseudomonas syringae* pv. *phaseolicola* strains, were kindly provided by Dr. Jesus Murillo (strain 1448A, race 6) and Dr Maria Holeva (strain BPIC593, Benaki Phytopathological Institute Collection, Dr A. Alivizatos, 1976). All strains were stored in glycerol stock solution, at -80° C. To ensure optimal growth conditions and purity, all the ATCC strains were sub-cultured in Blood and Mac Conkey Agar plates (Oxoid, UK) before each test. Accordingly, *P. syringae* pv. *phaseolicola* was cultured in Nutrient Broth Agar (PanReac, U.S.A.). The bacterial strains, the incubation conditions and the culture media used are given in Table 1.

Antimicrobial screening

For the antimicrobial assays, both in liquid and on solid bacterial cultures, the applied techniques assure repeatable results due to the continuously direct contact of EO with the bacteria**.** Laurel EO extracted from *L. nobilis* leaves was tested in the screening using two standard methods with agar plates: the Disc diffusion assay (DDA) and the Well diffusion assay (WDA). Moreover, the minimal inhibitory and the bactericidal concentration (MIC and MBC) of the EO in liquid medium were determined.

Inoculum suspension

The bacterial inoculum was prepared according to Clinical and Laboratory Standards Institute (2015) direct colony suspension method. Briefly, 24-hour bacterial colonies grown on solid media were suspended in sterile saline solution (0.9% NaCl) to achieve a turbidity of 0.5 McFarland (OD_{630}= 0,05) standard, corresponding to approximately 1 to 2×10^8 colony-forming units (CFU)/ml. The turbidity was measured using a spectrophotometer. For the broth dilution method, the inoculum (0.5 McFarland) was diluted 1:1000 in fresh LB broth for ATTC strains or NB for *P. syringae* pv. *phaseolicola* strains to obtain a concentration of $1\text{-}5\times10^5$ CFU/ml (National Committee for Clinical Laboratory Standards, 2015). The inoculum was used within 15 minutes from its preparation.

Table 1. The bacterial strains, culture media and growth conditions used in this study for the evaluation of antibacterial activity of the essential oil of *Laurus nobilis*.

Microorganisms	Culture media	Incubation conditions (time/temperature)
Pseudomonas syringae pv. *phaseolicola* BPIC593	Nutrient Broth Agar	24-48 hours/22 ˚C
Pseudomonas syringae pv. *phaseolicola* 1448A	Nutrient Broth Agar	24-48 hours/22 ˚C
Bacillus subtilis ATCC 9372	Blood Agar	24 hours/ 37˚C
Micrococcus luteus ATCC9341	Blood Agar	24 hours/ 37˚C
Staphylococcus aureus ATCC29213	Blood Agar	24 hours/ 37˚C
Escherichia coli ATCC25922	Mac Conkey Agar	24 hours/ 37˚C

Disc diffusion assay

Five hundred µl of the inoculum in saline solution (1-5×10^5 CFU/ml) was spread over the plates of pre-dried Mueller–Hinton agar (MHA, Oxoid) using a sterile cotton swab (National Committee for Clinical Laboratory Standards, 2015). It was allowed to dry for 1 min. Then, sterile filter paper discs of 6 mm diameter (Schleicher and Schuell, Dassel, Germany) were placed on the plates and immediately 8 µl of the *L. nobilis* EO extracted in this work were spotted. The negative and positive controls were sterile saline solution and antibiotics containing discs (gentamicin, 10 µg/ml for the ATCC strains and kanamycin, 50 µg/ml for the *P. syringae* pv. *phaseolicola* strains), respectively. The plates were incubated at 37°C for 24 h for the ATCC strains or at 22°C for 48 hours for the *P. syringae* pv. *phaseolicola* strains. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the paper disks. Each bioassay was carried out three times.

Well diffusion assay

Similarly to the procedure used in the Disc diffusion method, the Mueller–Hinton agar plate surface was inoculated by spreading 500 µl of the bacterial inoculum in saline solution (1-5×10^5 CFU/ml) over the entire agar surface. Then, a hole with a diameter of 6 mm was punched aseptically with a sterile tip and 8 µL of the *L. nobilis* EO extracted in this work was added into the well. Then, agar plates were incubated at 37°C for 20 hours for the ATCC strains or at 22°C for 40 hours for the *P. syringa*e pv. *phaseolicola* strains and the inhibition zones around the wells were measured.

Minimum Inhibitory Concentration (MIC)

The Tube Dilution Assay was performed as following: The MIC values of the EO were determined using a two-fold broth dilution procedure to get a concentration of the EO in the medium ranging from 5.0 to 0.156%, according to the Clinical and Laboratory Standards Institute M27-A3 guidelines (Clinical and Laboratory Standards Institute, 2008). Briefly, 20 µl of the EO were added to the first test tube containing 380 µl LB for the ATCC strains or Nutrient broth for the *P. syringae* pv. *phaseolicola* strains. Then, 200 µl from the first tube were transferred and serially diluted into the next tubes, which contained 200 µl of the same medium. This two-fold dilution was continued until the 10^{th} tube. The 200 µl from the 10^{th} tube were discarded. The 11th tube was used as the sterile control (negative control: the medium without bacterial inoculum) and the 12^{th} tube was reserved for the growth control (positive control: the medium with bacterial inoculum). Then, 200 µl of the bacterial inoculum containing 10^5 CFU/ml were added in tubes 1 to 10 and 12. All the tubes contained finally the same volume (400 µl) of medium. Ampicillin for the ATCC strains (100 µg/µl) or kanamycin (50 µg/µl) for *P. syringae* pv. *phaseolicola* strains were also used as positive antibacterial controls in an extra tube containing 200 µl of the antibiotic containing medium and 200 µl of the bacterial inoculum. Finally, tubes were incubated in the dark on an orbital shaker (160 rpm) at 37°C for 20 hours (Kavanaugh *et al*., 2012) for the ATCC strains or at 22°C for 40 hours for the *P. syringae* pv. *phaseolicola* strains. The lowest concentration of EO preventing visible growth (no turbidity) of a strain was identified as the MIC and expressed as % (v/v) of EO in culture medium. For *S. aureus*, 100 µl instead of just 20 µl of EO were also tested for antibacterial activity.

Minimum Bactericidal Concentration (MBC)

The complete absence of growth was considered as the MBC. To confirm the results of the MBC, 10 µL of the bacterial suspensions of the tube dilution assay (see 2.4.4), withdrawn from the tubes with little or no visible growth, were streaked on 1,5 % w/v LB-agar plates for the ATCC stains or 1.5 % w/v NB-agar plates for *P. syringae* pv. *phaseolicola* strains. Then the plates were incubated for 24 h at 37°C for checking of viable cells (National Committee for Clinical Laboratory Standards, 2015) for the ATCC strains or at 22°C for 48 hours for the *P. syrin-*

gae pv. *phaseolicola* strains. Total absence of bacterial colonies on the agar plate was determined as the MBC. Both the growth control (containing inoculum but no EO) and negative control (containing EO but not inoculum) were also plated on agar plates. Values were recorded as % (v/v) of EO in culture medium.

Statistical analysis

The mean values of the diameter (mm) of the inhibition zones ± standard deviations were calculated from three independent experiments.

Results

All the emulsifying agents used for the dilution of the laurel EO did not permit the production of a homogenous solution. Therefore, pure laurel EO, instead of diluted, was used for all the antibacterial assays.

Zones of inhibition

The zones of inhibition that were produced by the *L. nobilis* EO extracted from plants grown in the Greek area of the mountain Olympus are given in Table 2 and Figure 1. In the Disc diffusion assay, the EO inhibited the growth of all applied bacterial strains. Depending on the strain, the diameters of inhibition zones ranged from 7.5 to 20.8 mm. The EO exhibited the greatest antibacterial effect against the *M. luteus* with a diameter of inhibition zone 20.8±3 mm, followed by the *P. syringae* pv. *phaseolicola* that exhibited an inhibition zone of 15.0±3 mm (strain 1448A) and 14.0±3 mm (BPIC593 strain). A smaller inhibition zone was observed against *E. coli* (10.0±2 mm) and *S. aureus* (8.0±2 mm). The weaker antibacterial activity was noted against *B. subtilis* (7.5±2 mm). Negative control discs (saline solution) did not produce any zones of inhibition. Positive inhibition controls included: a) kanamycin disks (50μg/ml) that caused an inhibition zone of 19 mm for *P. syringae* pv. *phaseolicola* strain 1448A, and b) gentamicin disks (10μg/ml) that caused an inhibition zone of 23 mm for all other bacteria.

In the Well diffusion assay, the diameter of the inhibition zone for *M. luteus* was 18.0±3 mm and it was the biggest one. On the other hand, the smallest zone was noted for *E. coli* (9.0±3 mm), *B. subtilis* (8.0±3 mm) and *S. aureus* (8.1±2 mm). *P. syringae* pv. *phaseolicola* displayed a zone of 14.5±3 mm for strain 1448A and of 18.0±2 mm for the BPIC593 strain.

The MIC and MBC of the EO

The EO was tested by the broth dilution method to determine the MIC visually and the MBC by growth on agar medium, expressed as a percentage of % (v/v) of EO in the medi-

Table 2. Zones of bacterial growth inhibition of six bacterial strains by essential oil of *Laurus nobilis* using two agar diffusion methods.

Method	Diameter* of the zone of growth inhibition of six bacterial strains (mean of three replicates ± standard deviation in mm)					
	Pseudomonas syringae pv. *phaseolicola* (1448A)	*Pseudomonas syringae* pv. *phaseolicola* (BPIC593)	*Bacillus subtilis* ATCC 9372	*Micrococcus luteus* ATCC9341	*Staphylococcus aureus* ATCC29213	*Escherichia coli* ATCC25922
Disc diffusion assay	15.0±3	14.0±3	7.5±2	20.8±3	8.0±2	10.0±2
Well diffusion assay	14.5±3	18.0±2	8.0±3	18.0±3	8.1±2	9.0±3

*The diameter of the paper discs was 6 mm.

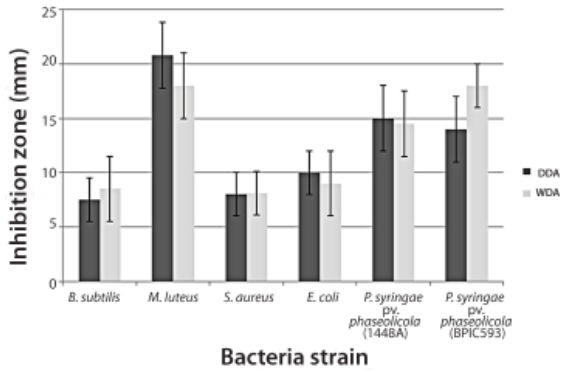

Figure 1. Histogram of inhibition zones (mm) measured in Disc diffusion assay (DDA) and Well diffusion assay (WDA) for the evaluation of the antibacterial activity of the essential oil of *Laurus nobilis* on six bacterial strains. Details are given in Table 2. Error bars represent the mean ± standard error from three independent experiments.

um. Since the addition of solvents and detergents in EO did not result in a homogenous solution, pure EO was used. As the antibacterial activity of the EO might be affected by the shaking rate and air exchange during culture (Wang *et al*., 2016), all liquid antibacterial assays were performed under constant agitation conditions on an orbital shaker (160 rpm) in tubes of 5 ml volume. By doing so, a direct-contact of bacteria and EO was achieved as well as the aeration of the media. Each experiment was performed three times. The results are summarized in Table 3. The EO was more potent against *M. luteus* (MIC and MBC were 1.25% and 2.5% respectively). The EO was also very effective against *P. syringae* pv. *phaseolicola* displaying a MIC of 1.25% for both strains and an MBC of 5% for the 1448A strain and 2.5% for the BPIC593 strain. The MIC for *B. subtilis* and *E. coli* were 2.5% and the MBC were 5% and 2.5%, respectively. On the other hand, the lowest antibacterial activity was displayed against *S. aureus,* since starting with 20 µl of EO in the first of the ten tubes for the antibacterial screening did not affect its growth. Therefore, 100 µl of EO were also used in the first tube instead of 20 µl. The MIC recorded was 1.563% and the MBC was 6.25%.

Table 3. Growth inhibitory effect of serially diluted essential oil of *Laurus nobilis* against six bacterial strains.

Bacterial strain		EO (v/v%)								
		5	2.5	1.25	0.625	0.3126	0.156	GrC[a]	StC[b]	SoC[c]
Pseudomonas syringae pv. *phaseolicola** (1448A)	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	+	+	+	+	+	+	-	-
Pseudomonas syringae pv. *phaseolicola** (BPIC593)	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
*Bacillus subtilis** ATCC 9372	MIC	-	-	+	+	+	+	+	-	-
	MBC	-	+	+	+	+	+	+	-	-
*Micrococcus luteus** ATCC9341	MIC	-	-	-	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
*Escherichia coli** ATCC25922	MIC	-	-	+	+	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-
		12.5	6.25	3.125	1.563	0.781	0.391	GrC[a]	StC[b]	SoC[c]
*Staphylococcus aureus*** ATCC29213	MIC	-	-	-	-	+	+	+	-	-
	MBC	-	-	+	+	+	+	+	-	-

a: Growth control (tube containing bacteria), b: Sterile control (tube without bacteria), c: Solvent control,
-: no growh, + : growth
* *B. subtilis*, *M. luteus*, *E. coli* and *P. syringae* pv. *phaseolicola* were incubated with 20 µL EO in the 1st tube
** *S. aureus* was incubated with 100 µL (20 µL EO did not inhibit its growth).

Discussion

Many EOs extracted from AMPs have been reported to have antimicrobial properties (Horváth and Ács, 2015). Therefore, *in vitro* studies including EOs which have been used in traditional medicines from the medieval times are important to improve antimicrobial treatments. EOs have unique metabolic profile (co-existence of phenols, flavonoids, alkaloids, terpenes, tannins etc) and exhibit their activity by many different inhibition mechanisms. Consequently, they can affect a variety of pathogens by changing membrane permeability, denaturing proteins and inhibiting enzymes (Nazzaro *et al*., 2013).

Standardizing the experimental methods for the bioassays

Testing plant EOs in liquid antimicrobial bioassays encounters problems mainly due to the EOs' water insolubility and complexity, i.e., a non-homogenous distribution of the oil through the medium appears and gives bacteria a greater chance of survival. To avoid these difficulties, many researchers propose the use of different solvents (methanol, ethanol, DMSO, Tween) (Wang *et al*., 2016). Unfortunately, the chemical properties of EOs do not permit a standardized methodology to dissolve them and the appropriate method depends on the EOs' nature. Laurel EO in this work was one of these cases, i.e., the low dispersion of water insoluble compounds of EO in the liquid growth medium (Tan and Lim, 2015). Different solvents and detergents (DMSO and Tween20) were tested. The dilution of laurel EO in all emulsifying agents resulted in a white 'color' suspension when the culture medium was added. The presence of the suspension did not permit the determination of MIC. The suspension might be caused by the lipophilic molecules of the laurel EO which form micelles, thereby suppressing EO's attachment to the microorganisms. These hydrophobic molecules interact with the lipids of the bacterial cell membrane, disturb cell structures and render them more permeable (Chouhan *et al*., 2017).

Only by using pure laurel EO with continuous shaking during incubation could we observe the antibacterial activity. Thus, for the *in vitro* investigation of the laurel EO antimicrobial activities in liquid culture, shaking could prevent the adherence clumping and phase separation between aqueous phase and EO. Additionally, Juergensmeyer *et al.* (2007) reported that shaking causes a decreased lag phase and a higher final yield. Our method is based on the National Committee for Clinical Laboratory Standards recommended standards for broth dilution assays. Moreover, the use of pure EO (without solvent) enhances the antibacterial activity. Undoubtedly, modifications should be made on proposed techniques since not all the EOs have the same diffusibility and solubility in liquid cultures (Rios *et al*., 1988). In field experiments, a totally different approach has to be applied. In this case, the EO/water dilution mixture would be directly sprayed on leaves.

In order to better evaluate the antimicrobial effectiveness of laurel EO, two different methods were applied, the Disc diffusion and the Well diffusion assay. The Disk diffusion assay is accepted by the FDA (Food and Drug Administration of the USA) and is established as a standard assay for the analysis of antimicrobial activity by the National Committee for Clinical Laboratory Standards (2015). The Well diffusion assay offers a higher sensitivity, faster and better diffusion of the EO into the agar (Valgas *et al*., 2007). Both antimicrobial assays resulted in similar results. We concluded that the Disc diffusion assay is an easier and less demanding technique than the Well diffusion assay. In the Well diffusion assay the main disadvantage is the difficulty in making the well.

In addition to the method of agar plates, a broth dilution method was used to determine the MIC of EO against the tested bacteria. The main advantage of MIC is the small quantity of reagents and samples required, which enables a greater number of repetitions and, thus, increases the reliability of the results. MBC was also evaluated, which is reported to be helpful in severe infectious

diseases (Nemeth *et al*., 2015).

Antibacterial properties of laurel EO

This work aims to perform an initial screening of the antibacterial properties of the specific EO, with emphasis on its properties over a plant pathogen. Data from our study has confirmed the antimicrobial potential of EO of *L. nobilis*. Furthermore, this is the first report regarding the antibacterial effect of laurel EO on the growth of two stains of *P. syringae* pv. *phaseolicola*, the well-studied 14484A and the strain BPIC593 isolated from plants grown in the Regional Unit of Florina, Greece.

P. syringae pv. *phaseolicola* is an important plant pathogen that mainly affects *P. vulgaris* crop production (Arnold *et al*., 2011). From our results it is well documented that the EO has a significant effect on the *P. syringae* pv. *phaseolicola* growth. Therefore, this study, together with previous research work by Bozkurt *et al.* (2020) on EO of *L. nobilis* against gall-forming phytopathogenic bacteria, establishes the grounds for the potential use of laurel EO as natural compounds alternative to chemically synthesized plant bactericides, whose use has already been deemed problematic (Lamichhane *et al*., 2015). Moreover, other EOs have been shown to exhibit *in vitro* antibacterial activity against *P. syringae* pv. *phaseolicola*, e.g. EO of *Origanum* species (Della Pepa *et al*., 2019). As there are increasing efforts in research to combat phytopathogenic bacteria with natural agents, we should note that most relevant studies employ culturing methods to assess the antibacterial effect of EOs on phytopathogenic bacteria and do not consider the viable but non culturable condition (VBNC) that may occur with bacterial cells. Therefore, in order to assess such phenomena, future studies should also incorporate viability testing using additional methods e.g., RT-PCR.

The EOs of *L. nobilis* growing in different countries have been reported to display antibacterial activity. Moghtader and Farahmand (2013) and Ouibrahim *et al.* (2013) noted antibacterial activity of laurel EO (by the Disc diffusion assay) against *E. coli*. In our screening, we noticed a rather small inhibitory activity of laurel EO towards *S. aureus*. This finding is in agreement with the results of Bennadja *et al.* (2013). Interestingly, even though *M. luteus* is a notoriously pathogenic microorganism in skin acne (Chiller *et al*., 2001) and *L. nobilis* EOs are used in cosmetics (Sahin Basak and Candan 2013), we found only few papers investigating *L. nobilis* EOs' antibacterial activity against *M. luteus*. In our work, we noticed the largest zone of inhibition for *M. luteus*.

Being an AMP, *L. nobilis* biosynthesizes a variety of active secondary metabolites. There are numerous phytochemical investigations on Mediterranean *L. nobilis* EOs. According to their results, the major constituents in laurel EOs are 1.8-cineole (eucalyptol), linalool and a-terpinyl acetate (Saab *et al*., 2012; Sahin Basak and Candan 2013). They are terpenoids and belong to the chemical subclass of oxygenated monoterpenes. 1,8-cineole is the major chemical component in leaf-derived EOs for the majority of medicinal species of *Eucalyptus* sp. followed by a-terpinyl acetate (Silva *et al*., 2011). Along the same line, Stefanova *et al.* (2020) have found in EO from Greek *L. nobilis* leaves the highest content in 1,8-cineole (30.8% of total ion current), but also a high content of α-terpineol (8%) and sabinene (7.9%) which belong to the oxygenated monoterpenes and monoterpene hydrocarbons, respectively. Other studies report the antimicrobial activity of these metabolites, such as linalool and 1,8-cineole. Sato *et al.* (2007) noted the highest antimicrobial activity for 1,8-cineole. According to the results by Hendry *et al.* (2009), a synergistic antimicrobial activity was noted for 1,8-cineole and chlorhexidine digluconate. The antimicrobial activity of 1,8-cineole might be attributed to the reduction of cellular activity by interacting with membrane enzymes and proteins (Stojković *et al*., 2011). Thus, the high content of oxygenated monoterpenes may, in a synergistic effect with minor compounds, provide the high antimicrobial activity of the Greek *L. nobilis* EO.

Conclusions

The current study reports, for the first time, the antibacterial activity of the Greek medicinal plant *L. nobilis* assessed by different *in vitro* assays against the plant pathogen *P. syringae* pv. *phaseolicola* and four human pathogenic bacterial species. Its EO is a valuable source of bioactive compounds that apparently have antimicrobial properties that inhibit the growth of different strains of the Gram-positive and Gram-negative bacteria tested. The chemical characterization of individual compounds could further shed light on the most effective combination that can be used as an antimicrobial agent. In conclusion, in a world of increasing antibiotic resistance of pathogenic bacteria, where there is an urgent need for new bioactive compounds, this initial step sets the basis for future use of natural agents of low cost and low environmental imprint to control bacterial plant and human pathogens.

We thank Mellifora for supplying the L. nobilis EO, Dr Maria Holeva (Laboratory of Bacteriology, Benaki Phytopatholigical Institute, Greece) for providing us with the BPIC593 strain of P. syringae pv. phaseolicola and Dr Jesus Murillo (Laboratorio de Fitobacteriologia, Institute for Multidisciplinary Research in Applied Biology, Universidad Publica de Navarra, Spain) for the P. syringae pv. phaseolicola strain, 1448A.

Declarations

Authors' contributions

S.M., A.G. and A.P. conceived and designed the experiments. S.M. and A.G. executed the experiments. S.M., A.G. and A.P. discussed the results and wrote the paper.

Funding

This research is co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Reinforcement of Postdoctoral Researchers - 2nd Cycle" (MIS-5033021), implemented by the State Scholarships Foundation (IKY).

Conflict of interest

The authors declare that there are no conflicts of interest.

Literature cited

Arnold, D.L., Lovell, H.C., Jackson, R.W. and Mansfield, J.W. 2011. *Pseudomonas syringae* pv. *phaseolicola*: from 'has bean' to supermodel. *Molecular Plant Pathology*, 12: 617–627.

Bennadja, S. Tlili-Ait-kaki, Y. Djahoudi, A. Hadef, Y. Chefrou, A. 2013. Antibiotic activity of the essential oil of laurel (*Laurus nobilis* L.) on eight bacterial strains. *Journal of Life Sciences*, 7: 814–819.

Bouzouita, N., Nafti, A., Chaabouni, M.M., Lognay, G.C., Marlier, M., Zghoulli, S. and Thonart, P. 2001. Chemical compositions of *Laurus nobilis* oil from Tunisia. *Journal of Essential Oil Research*, 13: 116–117.

Bozkurt, A., Soylu, S., Kara, M. and Soylu, E. 2020. Chemical composition and antibacterial activity of essential oils isolated from medicinal plants against gall forming plant pathogenic bacterial disease agents. *KSU Journal of Agriculture and Nature*, 23: 1474-1482.

Chiller, K., Selkin, B.A. and Murakawa, G.J. 2001. Skin microflora and bacterial infections of the skin. *Journal of Investigative Dermatology Symposium Proceedings*, 6: 170–174.

Chouhan, S., Sharma, K. and Guleria, S. 2017. Antimicrobial activity of some essential oils-present status and future perspectives. *Medicines* (Basel), 84(3): 58.

Clinical and Laboratory Standards Institute (2015) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.* Approved Standard-10th Edition. CLSI document M07-A10. Wayne, PA.

Clinical and Laboratory Standards Institute 2008. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Approved Standard-3rd Edition. CLSI document M27-A3. Wayne, PA.

Della Pepa, T., Elshafie, H. S., Capasso, R., De Feo, V., Camele, I., Nazzaro, F., et al. 2019. Antimicrobial and phytotoxic activity of *Origanum heracleoticum* and *O. majorana* essential oils growing in cilento (Southern Italy). *Molecules*, 24: 1–16.

Dioscurides, 77 AC. 2001. *De Materia Medica*. Militos

Publications, Athens, Greece, ISBN 960-8033-01-02.

Elshafie, H.S. and Camele, I. 2017. An overview of the biological effects of some mediterranean essential oils on human health. *BioMed Research International*, 2017:9268468.

Flamini, G., Tebano, M. Cioni, P. Ceccarini, L. Simone, S. and Longo, I. 2007. Comparison between the conventional method of extraction of essential oil of *Laurus nobilis* L. and a novel method which uses microwaves applied in situ, without resorting to an oven. *Journal of Chromatography*, 1143:36–40.

Georghiou, K. and Delipetrou, P. 2010. Patterns and traits of the endemic plants of Greece. *Botanical Journal of the Linnean Society*, 162: 130–422.

Giesecke, A. 2014. *The Mythology of Plants: Botanical Lore from Ancient Greece and Rome.* Getty Publications.

Hendry, E.R., Worthington, T., Conway, B.R. and Lambert, P.A. 2009. Antimicrobial efficacy of eucalyptus oil and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures. *Journal of Antimicrobial Chemotherapy*, 64: 1219–1225.

Horváth, G. and Ács, K. 2015. Essential oils in the treatment of respiratory tract diseases highlighting their role in bacterial infections and their anti-inflammatory action: A review. *Flavour and Fragrance Journal*, 30: 331–341.

Huergo, H. and Retamar, J. 1978 El aceite esencial de bay (*Laurus nobilis* L*). Rivista Italiana EPPOS*, 60: 635- 637.

Hussein, R.A. and El-Anssary, A.A. 2018. Plants secondary metabolites: the key drivers of the pharmacological actions of medicinal plants. *Herbal Medicine*, IntechOpen, London. 10.5772/intechopen.76139.

Juergensmeyer, M.A., Nelson, E.S. and Juergensmeye, E.A. 2007. Shaking alone, without concurrent aeration, affects the growth characteristics of *Escherichia coli*. *Letters in Applied Microbiology*, 45:179–183.

Kavanaugh, N.L and Ribbeck, K. 2012. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Applied and Environmental Microbiology*, 78: 4057–4061.

Kilic, A., Kollmannsberger, H. and Nitz, S. 2005. Glycosidically bound volatiles and flavor precursors in *Laurus nobilis* L. *Journal of Agricultural and Food Chemistry*, 53: 2231–2235.

Kupeli, E., Orhan, I. and Yesilada, E. 2007. Evaluation of some plants used in turkish folk medicine for their anti-inflammatory and antinociceptive activities. *Journal of Pharmaceutical Biology*, 45(7): 547–555.

Lamichhane, J.R., Messéan, A. and Morris, C.E. 2015. Insights into epidemiology and control of diseases of annual plants caused by the *Pseudomonas syringae* species complex. *Journal of General Plant Pathology*, 81: 331–350.

Mamoucha, S., Tsafantakis, N., Fokialakis, N. and Christodoulakis, N.S. 2018. A two-season impact study on *Globularia alypum*: adaptive leaf structures and secondary metabolite variations. *Plant Biosystems*, 152(5): 1118–1127.

Moghtader, M. and Farahmand, A. 2013. Evaluation of the antibacterial effects of essential oil from the leaves of *Laurus nobilis* L. in Kerman Province. *Journal of Microbiology and Antimicrobials*, 5: 13–17.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B. and Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature*, 403: 853–858.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. and Vincenzo, D.F. 2013. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* (Basel), 6: 1451–1474.

Nemeth, J., Oesch, G. and Kuster, S.P. 2015. Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*, 70: 382–395.

Ouibrahim, A., Tlili-Ait-kaki, Y., Bennadja, S., Amrouni, S., Djahoudi, A.G. and Djebar, M.R. 2013. Evaluation of antibacterial activity of *Laurus nobilis* L., *Rosmarinus officinalis* L. and *Ocimum basilicum* L. from Northeast of Algeria. *African Journal of Microbiology Research*, 7: 4968–4973.

Pino, J. Borges and P. Roncal, E. 1993. The chemical composition of laurel leaf oil from various origins. *Molecular Nutrition and Food Research*. 37: 592–595.

Politeo, O., Jukic, M. and Milos Ml. 2007. Chemical composition and antioxidant activity of free volatile aglycones from laurel (*Laurus nobilis* L.) compared to its essential oil. *Croatica Chemica Acta*, 80: 121–126.

Ramos, C., Teixeira, B., Batista, I., Matos O., Serrano, C., Neng, N.R., Nogueira, J.M.F., Nunes, M.L. and Marques, A. 2012. Antioxidant and antibacterial activity of essential oil and extracts of bay laurel *Laurus nobilis* Linnaeus (Lauraceae) from Portugal. *Natural Product Research*, 26: 518–29.

Rios, J.L., Recio, M.C. and Villar, A. 1988. Screening methods for natural products with antimicrobial activity: A review of the literature. *Journal of Ethnopharmacology*, 23: 127–149.

Saab, A.M., Tundis, R., Loizzo, M.R., Lampronti, I., Borgatti, M., Gambari, R., Menichini, F., Esseily, F. and Menichini, F. 2012. Antioxidant and antiproliferative activity of *Laurus nobilis* L. (Lauraceae) leaves and seeds essential oils against K562 human chronic myelogenous leukaemia cells. *Natural Product Research*, 26: 1741–1745.

Sahin Basak, S. and Candan, F. 2013 Effect of *Laurus*

nobilis L. essential oil and its main components on α-glucosidase and reactive oxygen species scavenging activity. Iranian *Journal of Pharmaceutical Research*, 2: 367–379.

Sato, K., Krist, S. and Buchbauer, G. 2007. Antimicrobial effect of vapours of geraniol, (R)-(-)-linalool, terpineol, γ-terpinene and 1,8-cineole on airborne microbes using an airwasher. *Flavour and Fragrance Journal*, 22: 435–437.

Silva, S.M., Yae Abe, S., Murakami, F.S., Frensch, G., Marques, F.A. and Nakashima, T. 2011. Essential oils from different plant parts of *Eucalyptus cinerea* F. Muell. ex Benth. (Myrtaceae) as a source of 1,8-cineole and their bioactivities. *Pharmaceuticals*, 12: 1535–1550.

Stace, C.A. 2010. New Flora of the British Isles (3rd edition). Cambridge, U.K.: Cambridge University Press. ISBN 9780521707725.

Stefanova, G., Girova, T., Gochev, V., Stoyanova, M., Petkova, Z., Stoyanova, A. and Zheljazkov, V.D. 2020. Comparative study on the chemical composition of laurel (*Laurus nobilis* L.) leaves from Greece and Georgia and the antibacterial activity of their essential oil. *Heliyon*, 6:e05491.

Stojković, D., Soković, M., Glamočlija, J., Džamić, A., Ćirić, A., Ristić, M. and Grubišić, D. 2011. Chemical composition and antimicrobial activity of *Vitex agnus-castus* L. fruits and leaves essential oils. *Food Chemistry*, 128: 1017–1022.

Tan, J.B.L. and Lim, Y.Y. 2015. Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. *Food Chemistry*, 172: 814–822.

Valgas, C., Machado, S., Elza, S. and Smânia, Jr. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, 38: 369–380.

Wang, T.H., Hsia, S.M., Wu, C.H., Ko, S.Y., Chen, M.Y., Shih, Y.H. et al. 2016. Evaluation of the antibacterial potential of liquid and vapor phase phenolic essential oil compounds against oral microorganisms. *PLoS One*, 11: e0163147.

Received:28 July2022; Accepted:4 January 2023

Αξιολόγηση της αντιβακτηριακής δράσης αιθέριου ελαίου δάφνης, *Laurus nobilis*, έναντι του βακτηρίου *Pseudomonas syringae* pv. *phaseolicola,* και πιθανής βιοκτόνου δράσης

Σ. Μαμούχα, Α. Προμπονά και Α. Γαλέου

Περίληψη Με τη συνεχή αύξηση του ανθρώπινου πληθυσμού στον πλανήτη μας, η επιβάρυνση του περιβάλλοντος από χημικά επιδεινώνεται συνεχώς. Επιπλέον, μεγάλο πρόβλημα αποτελεί η εμφάνιση ανθεκτικότητας των μικροοργανισμών, όπως τα βακτήρια, σε ευρέως χρησιμοποιούμενες χημικές ουσίες. Ως εκ τούτου, η εφαρμογή βακτηριοκτόνων προϊόντων που μειώνουν τους κινδύνους ανάπτυξης ανθεκτικότητας, για το περιβάλλον και την ανθρώπινη υγεία είναι εξαιρετικά ωφέλιμη. Στην παρούσα εργασία εξετάσαμε το αιθέριο έλαιο που εξάγεται από φυτά του είδους *Laurus nobilis* L. (δάφνη), τα οποία καλλιεργούνται στις παρυφές του όρους Όλυμπος, για την αντιμικροβιακή του δράση έναντι δύο στελεχών του παθογόνου *Pseudomonas syringae* pv. *phaseolicola* που προσβάλλει το φυτό *Phaseolus vulgaris*, και παθογόνων βακτηρίων για τον άνθρωπο (βιοκτόνος δράση). Τα αποτελέσματά μας, που ελήφθησαν με καθιερωμένες μεθόδους, όπως η Well diffusion assay και η Disc diffusion assay, αποκαλύπτουν ότι το αιθέριο έλαιο δάφνης είναι πολύ αποτελεσματικός βακτηριοστατικός και βακτηριοκτόνος παράγοντας. Για πρώτη φορά αναφέρεται η δράση του αιθέριου ελαίου δάφνης ως αναστολέα της ανάπτυξης του φυτοπαθογόνου βακτηρίου *P. syringae* pv. *phaseolicola*. Έτσι, η παρούσα εργασία ανοίγει το πεδίο για πιο εκτεταμένες έρευνες σχετικά με τη χρησιμοποίησή του στη φυτοπροστασία. Επιπλέον, το αιθέριο έλαιο δάφνης που δοκιμάστηκε έδειξε σημαντικές αντιβακτηριακές ιδιότητες έναντι πολλών παθογόνων βακτηρίων για τον άνθρωπο, όπως τα *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* και *Bacillus subtilis*.

Hellenic Plant Protection Journal **16:** 29-39, 2023

Τόμος 16, Τεύχος 1, Ιανουάριος 2023
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Περιεχόμενα

Volume 16, Issue 1, January 2023
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Hellenic Plant Protection Journal

Contents

www.hppj.gr