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ACTIVITY OF THYME ESSENTIAL OILS AGAINST PLANT PATHOGENIC FUNGI AND ITS POTENTIAL USE AS SEED DISINFECTANT

Denisa-Ioana GHEORGHE[1, 2#], Victoria BÎNZARI[1#], Diana CONSTANTINESCU-ARUXANDEI[1], Carmen LUPU[1], Sergey SHAPOSNIKOV[4], Florin OANCEA[1, 3]

[1]National Institute for Research & Development in Chemistry and Petrochemistry - ICECHIM, 202 Splaiul Independenței, District 6, 060021, Bucharest, Romania
[2]University of Bucharest, Faculty of Biology, Spl. Independentei, 91-95, District 6, Bucharest, Romania
[3]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[4]Norgenotech, Totenvegen 2049, 2848 Skreia, Norway
([#]Equal contribution)

Corresponding author emails: diana.constantinescu@icechim.ro, florin.oancea@icechim.ro

Abstract

One potential application of essential oils as low-risk biopesticides is as seeds disinfectant. The main goal of this study was to test the activity of thyme essential oil on four strains of seed/soilborne plant pathogenic fungi, Fusarium graminearum, Botrytis allii, Rhizoctonia solani, and Pythium ultimum, and to evaluate the effect of the inhibitory doses on plant seed germination. The direct application of thyme essential oil on seeds demonstrates a lack of selectivity. The main effects were related to radicle architecture and development of the primary and lateral roots, both in monocotyl, sorghum (Sorghum saccharatum), and in the dicotyls garden cress (Lepidium sativum) and white mustard (Sinapis alba). One potential solution to the lack of selectivity of direct application of the essential oils is biofumigation, i.e., application by volatilization in the rhizosphere area. Our paper raises this issue of compatibility between the necessary doses to inhibit the plant pathogenic fungi and the effects on plant seeds. Formulation of essential oils as slow-release nanoformulation could reduce phytotoxicity while maintaining and even enhancing the activity against plant pathogens.

Key words*: essential oils, antifungal, fungistatic effect, fungicidal effect, seed germination inhibition.*

INTRODUCTION

Fungal infections change the physiology of plants, disrupting their normal functioning, reducing their growth, and sometimes causing their cell death. The control of the fungal diseases is done by applying fungicide, a specific class of pesticides (Tudi et al., 2021).
Synthetic pesticides are considered a real problem to the environment and human health, which has increased the interest in the development of biodegradable and non-toxic compounds (Angelini et al., 2003; De Almeida et al., 2010; Nicolopoulou-Stamati et al., 2016). One possible alternative to synthetic pesticides/ fungicides is represented by essential oils (EOs) that are known for their antimicrobial and insecticidal properties (Raveau et al., 2020). EOs are mostly used in medicine-aromatherapy, the food industry and lately started to be used in agriculture. EOs are a new category of crop protection agents due to their pathogen inhibitory effects, short actual action time and low environmental toxicity (Arraiza et al., 2018). Studies are being carried out on the use of essential oils as effective biofungicides (Krzyśko-Łupicka et al., 2019). In recent decades, EOs have also been studied to evaluate their use in weed control, mainly due to their allelopathic potential (Abd-ElGawad et al., 2021) and enhancement of crop productivity (Chrysargyris et al., 2020). The compounds with such properties could be used for the biological control of plant pathogens and weeds as natural pesticides and herbicides with less destructive effects on the environment. The effect of their biological action depends on their chemical composition and the dose of essential oils, as well as the sensitivity of phytopathogen strains.
Researchers have identified mono- and sesquiterpene-type hydrocarbons as inhibitors

of microbial plant pathogens (Raveau et al., 2020). Usually mono and sesquiterpenes such as phenols, alcohols, ethers, carbohydrates, aldehydes and ketones are the main constituents of essential oils, being responsible for their biological activity as well as their odor. Components such as thymol, carvacrol, and linalool have antimicrobial activity and are found mostly in the composition of volatile essential oils (Cimanga et al., 2002).

One possible application is to inhibit the fungal infection of seeds by applying EOs as seed treatment, but EOs should not negatively affect the seeds and plant development.

Thymus vulgaris and other species of the Lamiaceae family, is native of Mediterranean countries, growing abundantly over wide areas in France, Spain, Portugal, Italy, Algeria and Morocco. Moreover, it is also cultivated in other parts of Europe and North America, North Asia and Oceania (Porte and Godoy, 2008). In addition to its use in foods, *T. vulgaris* is a well-known herbal medicine that has been used for thousands of years to treat alopecia, dental plaque, dermatophyte infections, bronchitis, cough, inflammatory skin disorders, and gastrointestinal distress. The major constituents of commercial *T. vulgaris* essential oil are thymol (23-60%), γ-terpinene (18-50%), p-cymene (8-44%), carvacrol (2-8%), and linalool (3-4%) (Duke, 2017).

EOs from *Thymus* genera exert antifungal, bactericidal, phytotoxic, antiparasitic, and insecticidal activities (Li et al., 2019; Zeljkovic and Maksimovic, 2015). This extended range of biological activities encourage their exploration and potential use for plant protection and food preservation purposes (Raveau et al., 2020). Different EOs obtained from some thyme species have demonstrated allelopathic effects and could be used for weed control (Ghasemi et al., 2020). Thymol, 5-methyl-2-isopropyl-1-phenol, the major constituent of EOs from *Thymus* species was approved as biocide/pesticide in European Union and USA (Rathod et al., 2021).

In this study we investigated the application of thyme essential oil (TEO) as seed disinfectant and soil treatment. The main aim of this study was to test the activity of TEO on four plant pathogenic fungi, *Fusarium graminearum*, *Rhizoctonia solani*, *Pythium ultimum* and *Botrytis allii* and to evaluate the effect of fungal inhibitory doses on plant seed germination. Each of the tested fungal pathogens causes devastating diseases, with important economic losses (Katan, 2017). Their epidemiological cycle is dominated by the dominancy of the soilborne primary inoculum (Lucas, 2006). Conservative farming practices, e.g, no-tillage and soil coverage with plant residues increase soilborne pathogens (Bockus & Shroyer, 1998; Page et al., 2013). Disinfection by biofumigation of the rhizosphere areas reduces not only the incidence of the seedling blight, root rot and damping off (Baysal-Gurel et al., 2018), but also the late plant disease, i.e., head blight (Drakopoulos et al., 2020).

TEO could be used as biofumigant from slow-release nanoformulations, applied as soil treatment or seed dressing, to decrease primary inoculum of the soilborne pathogen and to reduce the severity of plant disease. However, the main issue is related to the phytotoxicity of the thymol, the principal component of TEO (Pinheiro et al., 2015). The results presented here aim to evaluate the directions for development of TEO nanoformulation with reduced phytotoxicity and even enhanced inhibitory activity against plant pathogens.

MATERIALS AND METHODS

Materials

The following culture media and chemicals were used: Potato Dextrose Agar (PDA, purchased from Scharlau, Barcelona, Spain); synthetic low-nutrient agar (KH_2PO_4 1.0 g/L, KNO_3 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KCl 0.5 g/L, glucose 0.2 g/L, sucrose 0.2 g/L, agar 15 g/L, all substances being for microbiological use, purchased from Scharlau, Barcelona, Spain), thyme essential oil, according to ISO 19817:2017, with 54% thymol (Aroma-Soap, Salonta, Romania); agar for roots proton pump assay (Scharlau, Barcelona, Spain) and bromo-cresol (Sigma, Merck Group, Darmstad, Germany); reagents for reactive oxygen species identification: Phosphate Buffer (Scharlau, Spain) and Nitroblue Tetrazolium Chloride, NBT (Sigma, Merck Group, Darmstad, Germany); phytotoxicity test kit according to ISO 18763:2016, Phytotox kit - for liquid

samples (supplied by MicroBioTests, Gent, Belgium) with 3 species of seeds included (*Sorghum saccharatum*; *Lepidium sativum*; *Sinapis alba*); pure water produced by Milli-Q® Purification System (Merck, Darmstad, Germany).

Fungal strains and cultivation methods

The fungal strains used in this study were *F. graminearum* CBS 454.97 (Culture collection of fungi and yeasts, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherland), *R. solani* anastomosis group AG-4, *P. ultimum* DSM 62987, *B. allii* DSMZ 62081 (DSMZ - German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany). The PDA medium was prepared following the standard protocol and it was sterilized in an autoclave (MLS - 3751L, PHCBI, Tokyo, Japan) at 121ºC for 15 minutes. The PDA medium was poured in sterilized 90 mm Petri dishes, 20 ml per dish. Fresh cultures of *F. graminearum*, *R. solani, P. ultimum* and *B. allii*, were prepared by inoculating mycelial disks excised from the edge of old cultures into the centre of Petri dish with PDA medium. The inoculated Petri dishes with the plant pathogenic fungi were kept in an incubator (MIR-154-PE, PHCBI, Tokyo, Japan), at 28ºC, for 7 days.

Fungistatic activity of thyme essential oil

Antifungal assays were performed using the filter paper disc diffusion method according to (Soylu et al., 2010) with minor modifications and applied on plate lid. Mycelium 6 mm discs of 10 days old fungus grown previously on synthetic low-nutrient agar were placed at the center of 60 mm Petri dishes containing PDA culture medium and incubated at 28˚C in darkness. Several doses of pure TEO were applied on sterilized filter paper disc (Whatman No. 1) of 5 mm diameter and placed on the inner surface of the inverted lid of Petri dishes. The plates were immediately sealed with parafilm to prevent the loss of essential oils from the plates and incubited at 28°C until the control was completely grown in the plate. The doses varied between 0.005 and 8.77 µL/cm^3. The mean radial mycelial growth of the pathogen was determined by measuring the diameter of the colony in two directions at right angles when the plate surface of the control was covered by fungus.

The growth inhibition rate (GIR), was measured as the percentage of inhibition of radial growth relative to the control, calculated using the following formula (eq. 1):

$$GIR = ((DC - DT) / DC) \times 100 \quad \textbf{(eq. 1)}$$

where: DC represents the mycelial growth diameter of the fungal colony in the control and DT represents the mycelial growth diameter in the treatment. Fungal growth was measured using ImageJ software. Experiments were performed in triplicate. The minimum fungistatic dose (MFsD) was the dose at which the growth inhibition was 100%.

Fungicidal activity of thyme essential oil

To establish the fungicidal effects of TEO on the phytopathogens of interest, transfer experiments were done. The mycelium discs that were 100% inhibited by TEO dose were transferred to fresh PDA to assess their viability after five days incubation at 28°C. The residual fungal growth was monitored by measuring the radial growth of the fungi and the minimum fungicidal dose (MFcD) was determined as the dose for which there was zero residual fungal growth.

The activity of thyme essential oil on plant seeds

The effect of TEO on plant seeds was determined using Phytotox kit at 0.1% TEO, approximately the average of the 3 highest MFcD obtained. The seeds were incubated in the provided plate with 20 mL ddH_2O (control) and 20 mL ddH_2O + 0.02 mL TEO. On each plate, 10 seeds were placed, at 10 mm from each other. The samples were incubated in the growth chamber for 3 days, in an upright position, in their cardboard supports, in a growth chamber at 25°C (+/- 1°C) for 3 days. Phytotoxicity of TEO comprised several procedures and measurements: percent of germinated seeds, roots and shoots length, roots proton pump, seedling qualitative and semi-quantitative total reactive oxygen species, by staining with nitro blue tetrazolium (NBT) and stereomicroscope analysis by Image Analysis software - ImageJ (Schindelin et al., 2015).

Percentage of germinated seeds, roots and shoots length

After the 3 days of seeds incubation, the plates were photographed and incubated in the climate chamber, for another 24 hours in the dark. Percentage of germinated seeds was calculated according to the formula:

$$((A-B) / A) \times 100 \qquad \textbf{(eq. 2)}$$

where: A-control and B-TEO treatment. The roots and shoots were measured by photographing the plates and performing analysis with ImageJ (Schindelin et al., 2015).

Proton pump assay

Roots proton pump assay was adapted according to the previous method (Zandonadi et al., 2016) with minor modifications.

The specific medium was prepared: 0.75% agar, bromocresol purple (0.004% /L). The pH was adjusted to 6.8 before the agar was added. The medium was autoclaved at 121°C for 15 minutes. After cooling, 25 mL of medium was poured into 90 mm Petri plate. For each Petri dish, 2 seedlings was positioned, with the roots embedded in the agar, and the stem and leaves outside the environment. The plates were incubated at room temperature for 24/48 h, depending on the plant species, after which they were examined.

Reactive oxygen species assay

The protocol for detection of O^{2-} ions in plant tissues with NBT was used (Kaur et al., 2016). Briefly, the roots were soaked for 15-20 min in eppendorf tubes containing 2 mL of 2 mM NBT in phosphate buffer pH 6.1. The roots were next washed with ddH_2O and visualized under stereomicroscope and the color intensity was analyzed with ImageJ.

RESULTS AND DISCUSSIONS

The growth of all the strains tested was inhibited by TEO, but the inhibitory dose was dependent on fungal species.

Figure 1A shows the inhibitory percent as a function of dose for *B. allii*, in the fungistatic test. The aspect of the inhibition of colonies growth and development at various TEO doses, is presented in Figure 1B.

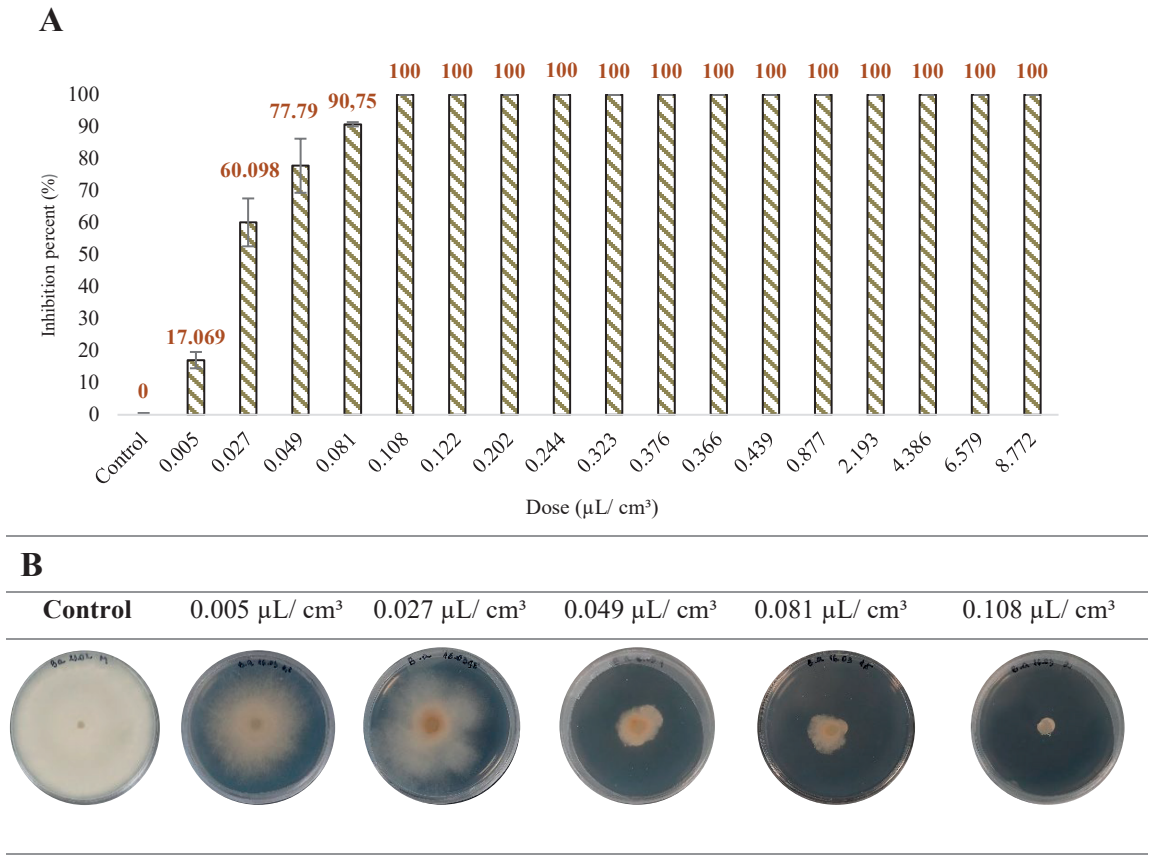


Figure 1. (A) Inhibition percent of mycelia growth of *B. allii* as a function of TEO dose; (B) Aspect of *B. allii* colonies in Petri dish at different TEO doses

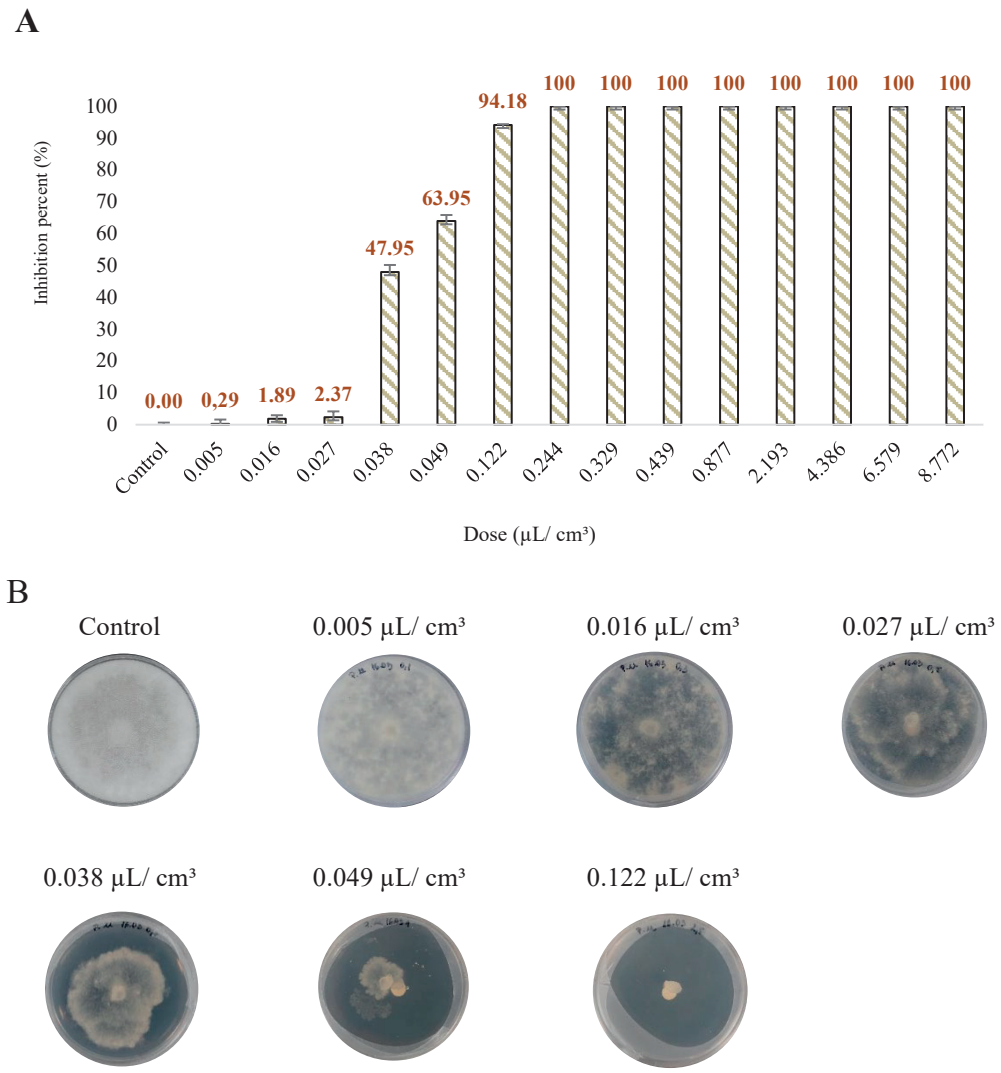


Figure 2. (A) Inhibition percent of mycelia growth of *P. ultimum* as a function of TEO dose; (B) Aspect of *P. ultimum* colonies in Petri dish at different TEO doses

The complete fungistatic effect was reached within the first 5 doses tested (MFsD 0.108 µL/cm^3). Similar behaviour was observed for the other strains, the most resistant strain being *P. ultimum* (Figure 2A and B, MFsD 0.244 µL/cm^3) and the least resistant being *R. solani* (MFsD 0.054 µL /cm^3).

The mycelium discs incubated at doses that induced 100% fungistatic effects were tested for MFcD of TEO, by transferring them on fresh PDA medium without TEO and evaluating the residual growth on fresh PDA medium after previous exposure to the doses with total fungistatic effect. Figure 3A and B shows the behaviour of mycelium *B. allii*, with a large difference between MFsD and MFcD.

All MFsD and MfcD values are included in Table 1. One can see that MFcD can be from much higher (20x higher MFcD than MFsD in the case of *B. allii*) to identical to MFsD (*R. solani* and *P. ultimum*), with intermediate difference between MFsD and MFcD for *F. graminearum*. The highest MFsD (*P. ultimum*) is 4*x* higher than the lowest MFsD (*R. solani*) and the highest MFcD (*B. allii*) is almost 40x higher than the lowest MFcD (*R. solani*).

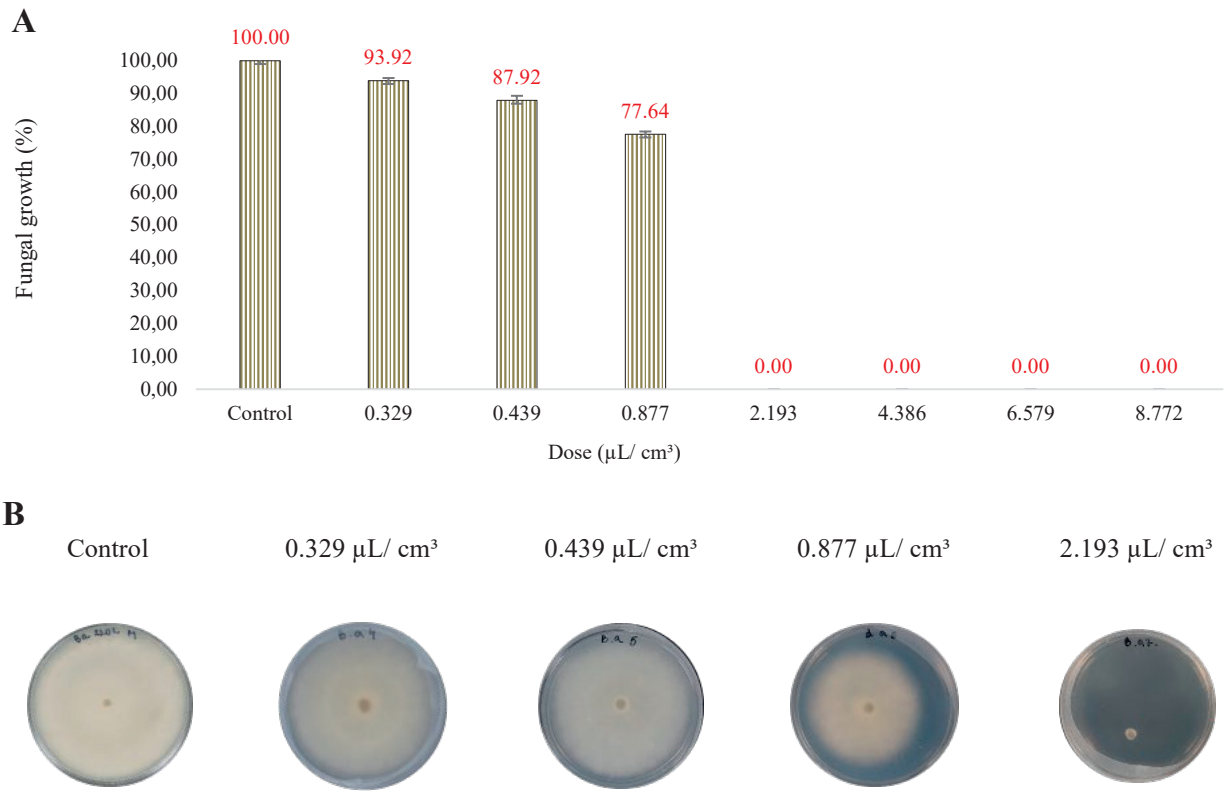


Figure 3. (A) Percent of residual growth of *B. allii* on fresh PDA after previous exposure to the dose represented on the X axis; (B) Aspect of the *B. allii* colonies up to MFcD (2.193 µL/ cm³)

Table 1. MFsD and MFcD values of TEO

Fungal strain	MFsD (µL/cm³)	MFcD (µL/cm³)
F. graminearum	0.122	0.439
B. allii	0.108	2.193
R. solani	0.054	0.054
P. ultimum	0.244	0.244

F. graminearum Schwabe (teleomorph: *Gibberella zeae*) is one of the most destructive plant pathogens, causing seedling blight, crown and root rot and head blight in cereals (Del Ponte et al., 2022). *F. graminearum* is a soilborne pathogen and agricultural practices are key factors in controlling its primary inoculum (Leplat et al., 2013).

R. solani Kühn (teleomorph: *Thanatephorus cucumeris*) is an ubiquitous soilborne plant pathogen that infects a wide range of important cultivated plants - cereals, legumes, potatoes, root crops such as sugar beet, fodder crop such as alfalfa, leafy vegetables (Ajayi-Oyetunde & Bradley, 2018).

P. ultimum is an oomycetes plant pathogen that produce seedling blight and root/stem rot on a wide range of host plants, including corn, vegetables and ornamentals such as flower bulbs, summer flowers and perennials (Kamoun et al., 2015). *P. ultimum* thrives in soil or substrates with high-water content and at medium temperatures and proliferates in no-tillage managed fields (Bickel & Koehler, 2021).

Plant pathogens from *Phytium*, *Fusarium* and *Rhizoctonia* spp. are the most frequent pathogens that cause damping-off of seedling, a devastating disease in nursery and greenhouses (Bickel et al., 2021).

B. allii was for long time considered either a synonym for *B. aclada* or a hybrid between *B. byssoidea x B. aclada* (Staats et al., 2004). Today is recognised as a distinct specie, (telemorph *Botryotinia allii*), closely related to *B. byssoidea* and *B. aclada* (Steentjes et al., 2021) *B. allii* is a pathogen that causes onion neck rot, survives in soil or on rotten bulbs as sclerotia. During storage, the infection is mostly seen in the throat region, but can also come from any tissue that comes in contact with the fungal inoculum (Steentjes et al., 2021).

As we mentioned already, we selected these fungal plant pathogen species because their soilborne primary inoculum is essential for

plant disease progression and biofumigation in the rhizosphere with EOs could be effective.

In order to test the effects on seeds, we applied the average dose of the 3 highest MFcD (0.941 µL/cm³, approximated to 0.1%) on 3 plant species: *Sorghum saccharatum*, *Lepidium sativum*, *Sinapis alba*.

Photos of the plants were taken in order to measure the roots and shoots length (Figure 4). TEO partially inhibited the seed germination and more significantly the development of seeds.

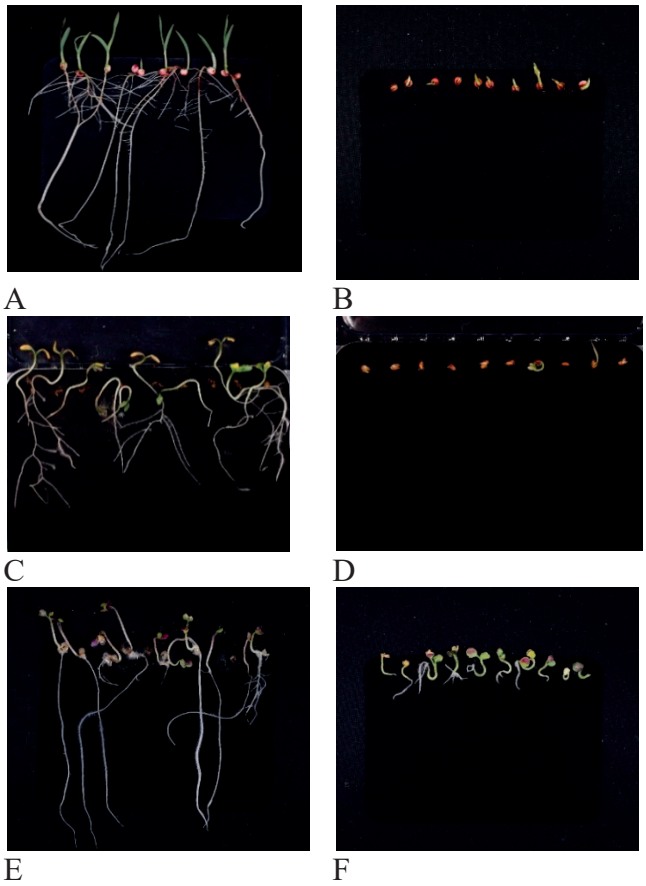


Figure 4. Seed germination and growth of *S. saccharatum* (A, B); *L. sativum* (C, D); *S. alba* (E, F) without (A, C, E) and with (B, D, F) TEO

The germination inhibition induced by TEO was 34% for *Lepidium sativum*, 25% for *Sorghum saccharatum* and 4% for *Sinapis alba.* The most significant phytotoxic effect of TEO was manifested as root growth inhibition: 97% (*L. sativum*), 87.11% (*S. alba*), 97.47% (*S. saccharatum*).

The development of primary and lateral root was affected, resulting in deterioration of root architecture. The shoot development was mainly affected in the case of *L. sativum* (almost 99%) (Figure 5).

The TEO effect was lower for *S. alba* seedling, formed from an oleaginous seed, than was for the other seedlings, formed by starchy seeds.

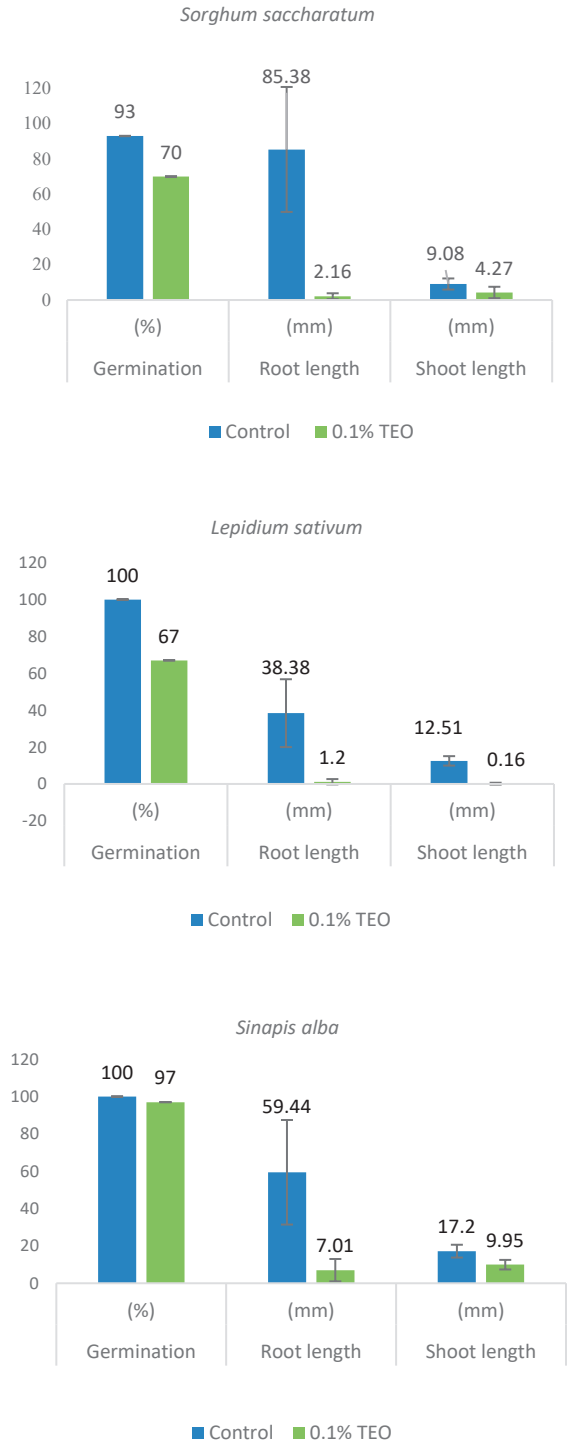


Figure 5. Germination percent, root length and shoot length of seeds in the presence and absence of 0.1% TEO

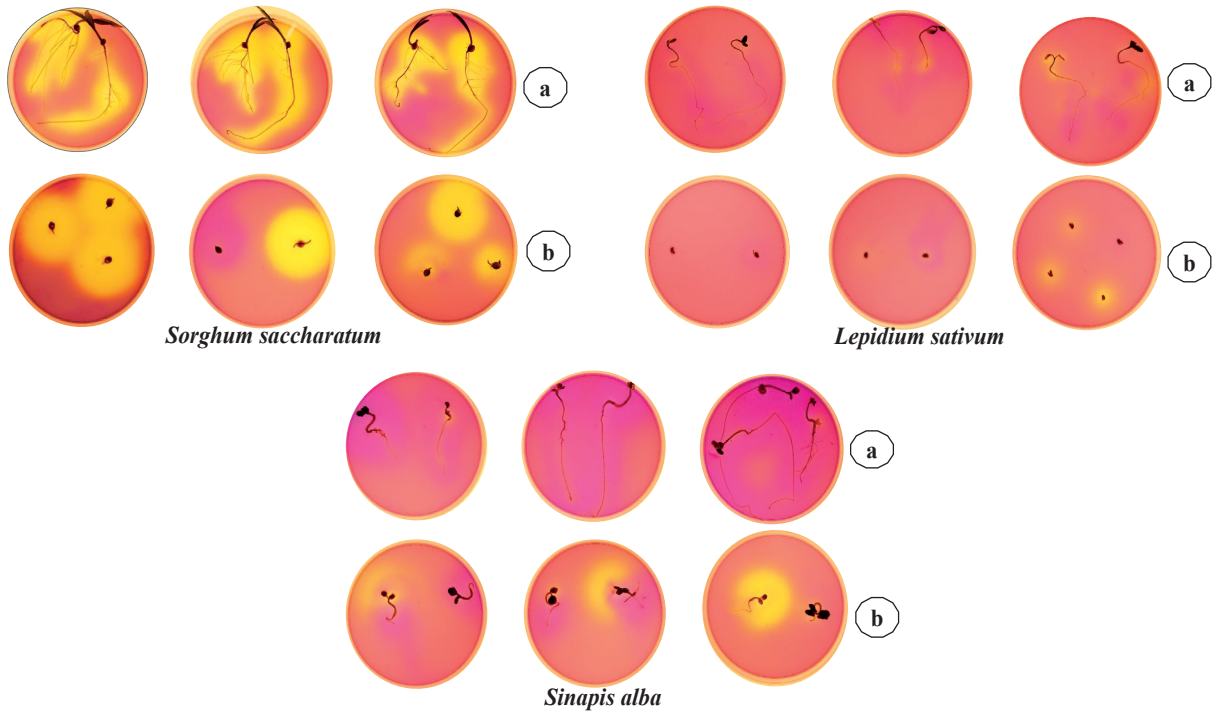


Figure 6. Proton pump from seedling of the tested plants not-exposed (a) and exposed (b) to the thyme essential oils

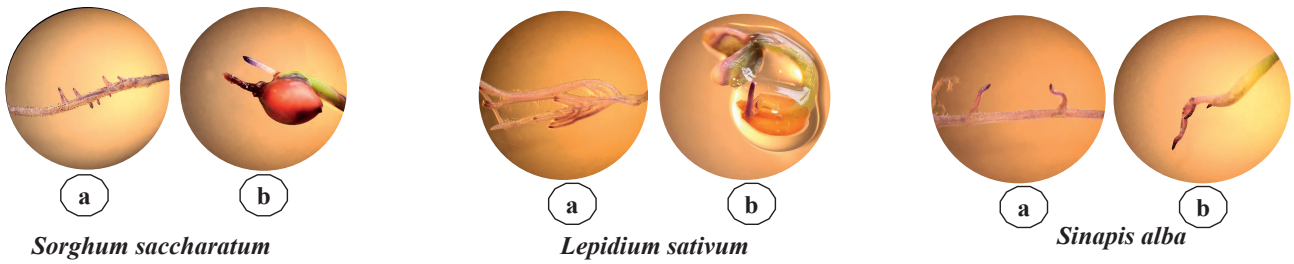


Figure 7. Detection of O^{2-} ions with nitro blue tetrazolium in tissues of plant not-exposed (a) and exposed (b) to the thyme essential oils

The oil bodies reduce toxic effect of hydrophobic TEO oil constituents. The same reduced toxic effect of TEO was determined for another oleaginous seed, soybean (Sotelo et al., 2021). The proton pump is slightly amplified in C3 seeds (*L. sativum*, *S. alba*) and is not affected in the C4 monocot seeds (*S. saccharatum*) by the TEO treatment (Figure 6). Most probably this is related to the main mechanism of EOs, including TEO phytotoxicity – induction of high level of reactive oxygen species (Lima et al., 2019; Singh et al., 2009; Staszek et al., 2021).

Intensity of the color formation by NBT in reaction with ROS, determined by image analysis of the radicles, suggest a modulation of the reactive oxygen species level during seed germination. In sorghum, a monocot plant, TEO have not a statistical influence on ROS formation, with tendency to decrease, In such monocot plant ROS play a significant role in seed germination (Farooq et al., 2021). In the dicot plant the ROS production is significantly amplified (Figure 7 and Figure 8). These dicot plants are more sensitive to endo-signals formed by ROS, such as modification of the redox status of glutathione pool (Castro et al., 2021).

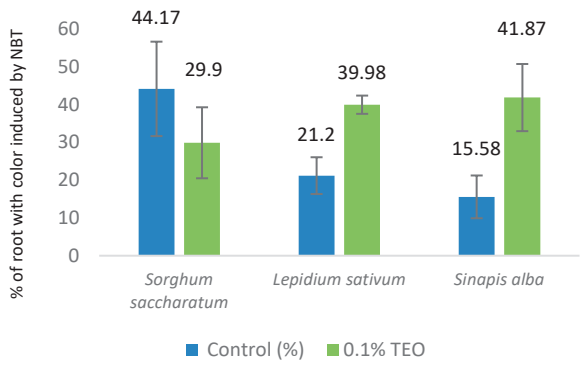


Figure 8. Intensity of the color formation by nitroblue tetrazolium in reaction with reactive oxygen species from radicles of the tested seedlings, determined by image analysis of the stained radicles.

The results presented here suggest that the formulation of the TEO should include hydrophobic compounds and a safener, a compounds that protect plants against ROS action. Such formulation will slowly release the volatile components and will reduce their side-effects.

CONCLUSIONS

TEO has both fungistatic and fungitoxic effect. The most sensitive fungal specie, both to fungistatic effects and to fungitoxic effects was *R. solani.* TEO has potential to reduce inoculum of soilborne plant pathogens by reducing the soil inoculum by fumigation.
TEO is phytotoxic for the tested seeds, *S. saccharatum*, *S. alba,* and *L. sativum*. The phytotoxic effect is related to higher production of ROS, Further studies should nanoformulate TEO with hydrophobic compounds and compounds that protect plants against ROS action.

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