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Allelopathic activity of thymol on seeds germination and effect on seedling properties at the non-allelopathic dose

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Abstract
The work responds to an important problem, concerning the use of thymol in biocontrol against pathogens and weeds in agriculture as an alternative to artificial chemical pesticides and herbicides. Experimentally, the tests are carried out on three types of seeds from three large crops; Wheat, Corn, and Lentils. Tests, *in vitro* and *in vivo*, of allelopathic activity on germination were carried out. Measurements of certain growth parameters of thymol-treated seedlings were determined *in vivo* after 15 days of growth under non-allelopathic conditions (non-allelopathic dose). Germination results obtained *in vitro* showed phytotoxic effects of thymol on the seeds of the three species of plants selected at low doses. The values recorded, *in vivo*, on the germination of the seeds of the three species confirm the non-allelopathic action of the concentration 0.05% thymol determined *in vitro*, while at this concentration, thymol influences the growth parameters, causing a decrease in biomass and photosynthetic pigments. In addition, the growth in length was not influenced, good growth in seedlings was observed. The results obtained have just demonstrated and confirmed the existence of the phytotoxic effects of thymol on the seeds and seedlings of three important crops for humans. It is therefore important to control the use of biopesticides in fields even at non-allelopathic doses of germination.

Keywords Agriculture, thymol, biocontrol

1. Introduction

Secondary plant metabolites such as terpenoids, steroids, phenols, coumarins, flavonoids, tannins, alkaloids and cyanogenic glycosides have been known to be involved in allelopathic phenomena and are important in all agroecosystems [1]. A number of these allelochemicals have been isolated and investigated to develop new biopesticides (insecticides, herbicides, fungicides) because, for many years, synthetic substances such as fungicides have been used for control plant pathogenic fungi and these chemicals have led to the development of resistance in many areas around the world [2] and many health problems such as cancer.

Essential oil and their major constituents, monoterpenes are among the most promising classes of natural products that can be used as safer pest and disease control agents [3]. Thymol is a monoterpene with antiseptic effects, found primarily in thyme, oregano, and tangerine peel [4]. Studies have shown that essential oil containing thymol is widely used in medicine for their antimicrobial, disinfectant properties [5-7] and several in vitro research have tested the toxic action of thymol and essential oil containing thymol against pathogenic fungi, including *Aspergillus flavus* [8-12].

According to researchers and scientists, thymol and/or other natural compounds with pesticidal properties are useful as alternative pest control agents [13-18]. A range of work has been done on essential oils and their majority compounds such as thymol as part of the protection of seeds and plants beneficial to humans from harmful microbes, molds and other protists, other studies have looked at weed control. They have also demonstrated the role and influence on the germination, growth, survival, and reproduction of these plants harmful to crops, but these works have not experimented in parallel the effects of these bioactive substances on cultivated species. If these bioactive compounds are safe, effective and biodegradable, their use in agricultural practice should take into consideration their allelopathic action. For this reason, to use thymol in the fields, for seeds and crop protection against pathogenic microorganisms and weeds, it is necessary to control its possible allelopathic effects.

For that, the aim of this study was to determine, *in vitro* and *in vivo*, the allelopathic effects of thymol on germination of some plant species of economic interest such as *Triticum aestivum*

L., *Zea mays* L., and *Lens culinaris* Medik. and its effects on the growth of seedlings as well as on the photosynthetic parameters.

2. Materials and Methods

2.1 *In vitro* seeds germination

Thymol ($C_{10}H_{14}O$; 99-100%) was purchased from Riedel de Haën, Germany. Solutions test with diverse concentrations of thymol were prepared by addition thymol in an aqueous solution containing 0.1% Tween 40. For control, only distilled water (T1) and 0.1% Tween 40 (T2) were used. Local seeds of common wheat, corn, and lentils were obtained from commerce. Seeds were surface sterilized by soaking them in 1% sodium hypochlorite solution for 5min, followed by several times rinsing in sterilized distilled water. One hundred of each type of seeds were introduced in 100ml solution test (or control solution) and kept for a time of immersion about 12 hours, then each 20 seeds were placed on a filter paper, moistened with sterilized distilled water, in a 10cm diameter Petri dishes, scaled with a paraffin film to prevent evaporation. All experiments were carried out at 25 °C in a germinator for 3 days. An emerged radicle was the criterion for germination. Seeds that showed radicle protrusion were considered germinated. The number of germinated seeds was counted and the percentage of germination (G) was calculated by equation (1):

$$G = \left(\frac{n}{N}\right) * 100 \qquad \dots (1)$$

Where

G: seeds germination in percentage.

n: number of seeds germinated.

N: total number of seeds.

2.2 *In vivo* germination and seedling growth

To study the effect of non Allelopathic dose of thymol; determined by *in vitro* test; on seedling growth and some growth parameters, The seeds of *Triticum aestivum* L., *Zea mays* L. and *Lens culinaris* Medik were sown in plastic recipients containing soil after soaked one night in 0.05% thymol solution. The seeds in a total of 100 were distributed in 5 pots; 20 seeds for each pot. In parallel, the control was realized without thymol. The treated and control seeds were watered every day and kept in sunlight. After the germination, the seeds sprouted were counted and the shoots in test pots were sprayed every day with 10ml of 0.05%thymol solution, while the shoots in control pots were sprayed every day with 10ml water.

Aerial parts length of plants was measured from the 5th day until the 15th day of growth by conventional method and the results were expressed in cm. On the last day of the experiment (15th day), the aerial parts of the seedlings are recovered for the measurement of water content, photosynthetic pigments, dry matter, and proteins. For chlorophylls and carotenoids content, fresh samples were used for extraction, using acetone 80% as solvent according to the method described by Pompelli *et al.* [19]. The absorbance at 663nm, 646nm, and 450nm was measured by spectrophotometer and the concentration of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were calculated using the following equations (2):

$$\begin{aligned} &Ch\ a=12.21Abs(663)- 2.81Abs(646)\\ &Ch\ b=22.13Abs(646)- 5.03Abs(663) \qquad (2)\\ &Ch\ t=Ch\ a+Ch\ b\\ &Carot. = (1000Abs(450) - 3.27Ch\ a - 104Ch\ b)/229 \end{aligned}$$

Where, $Ch\ a, Ch\ b, Ch\ b, Carot.$ are the chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid and Abs is the absorbance.

For dry matter and protein contents, plant biomass was obtained after drying at 105 °C for 30min and followed at 80 °C until constant weight has been attained. The protein amounts were determined, by the Bradford method, using the procedure described by Bonjoch and Tamayo [20].

2.3 Statistical analysis

All experiments, extractions and analyses were performed in triplicate for each sample, and the values of results were expressed as mean ± standard deviation (SD). Statistical evaluation of the findings was performed by analysis of variance (ANOVA) and Tukey's comparison test, and the value of $p \leq 0.05$ was considered significant.

3. Results and Discussion

3.1 *In vitro* seeds germination

The results showed any inhibitory effect of Tween 40 (0.1%) on seeds germination, and statistically, comparison with two control, revealed none effect on germination at the concentration of 0.05% thymol in all sample tests, but the toxic effect of thymol on seed starting to exhibit at the dose of 0.06% and evolves and progresses very quickly at low doses. These results announced that thymol has allelopathic activity and significantly inhibits seeds germination of wheat, corn, and lentils, confirming as well as data from the literature that say that the monoterpenes are very powerful germination inhibitors [21].

Figure 1 shows that the seeds of wheat and lentils are very sensitive than corn seeds. Thymol at 0.13% reduced completely germination of seeds from *Triticum aestivum* L. and *Lens culinaris* Medik. While treatment at the same concentration (0.13%); thymol reduced *Zea mays* L. seeds germination by 80%, only 20% of seeds have germinated. Total germination inhibition of corn seeds was observed at the concentration of 0.25%.

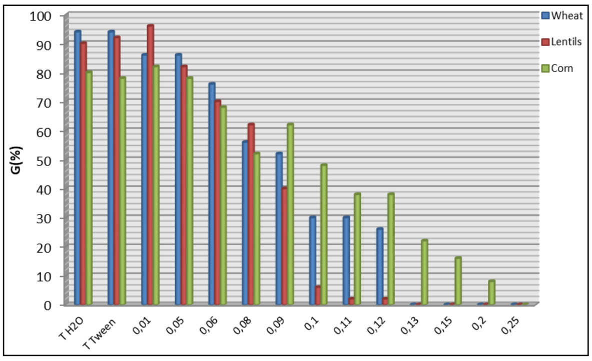

Fig 1: *In vitro*, effect of different concentrations of thymol on *Triticum aestivum*, *Lens culinaris* and *Zea mays* seeds germination, compared with two controls (T: control)

3.2 *In vivo* germination and seedling growth

The tests in this experiment were conducted with thymol under non-allelopathic conditions of germination.

3.2.1 Seed germination

Germination increased to 75-92% at ambient temperature (figure 2). The highest germination rate was observed in wheat control (92%). The germination was observed until the 7th day after no germination was observed.

In all three types of seeds, controls show a slightly high germination rate at treated seeds. Statistically, this tiny difference between controls and treated is not significant. Comparing seed germination between samples. control with control and treated with treated, we find that wheat seeds sprout much more than the seeds of the other two samples and that lentil seeds germinate less than other types and have a slightly low germination rate (81% for controls and 75% for treated) compared to wheat and corn seeds.

The results obtained in this test confirm those obtained in the first previous test (*in vitro*). It is concluded that the dose of 0.05% thymol is non-allelopathic dose and the germination of the seeds treated with thymol under *in vivo* conditions is statistically similar to the control seeds.

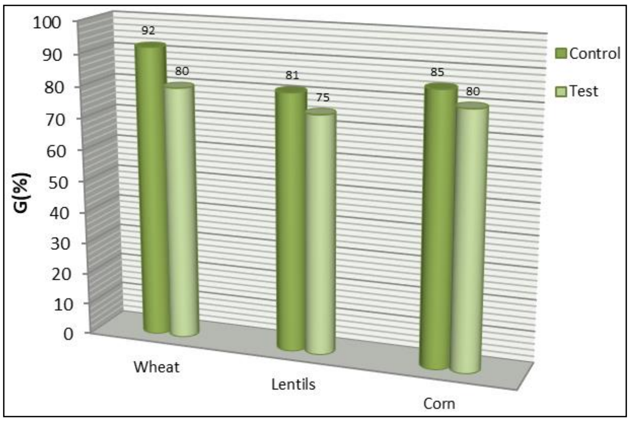

Fig 2: *in vivo*, effect of 0.05% thymol on germination seeds

3.2.2 Seedling growth and biomass

Seedling growth parameters are measured at the non-allelopathic dose (0.05%). The young plants were detached from the soil, the roots removed and the aerial parts were weighed and the length measured. The effects of thymol on seedling growth in terms of length after 15 days of treatment are shown in figure 3. The shoot length of various samples was ranged from 14.47cm for treated corn to 22.79cm for treated lentil and 14.41cm for untreated wheat to 18.13cm for untreated corn. Based on the recorded data, we found that thymol does not affect the shoots length growth of wheat and corn samples, in contrast very good growth is observed for samples of lentils treated with thymol (22.79cm) compared to control shoots (18.13cm).

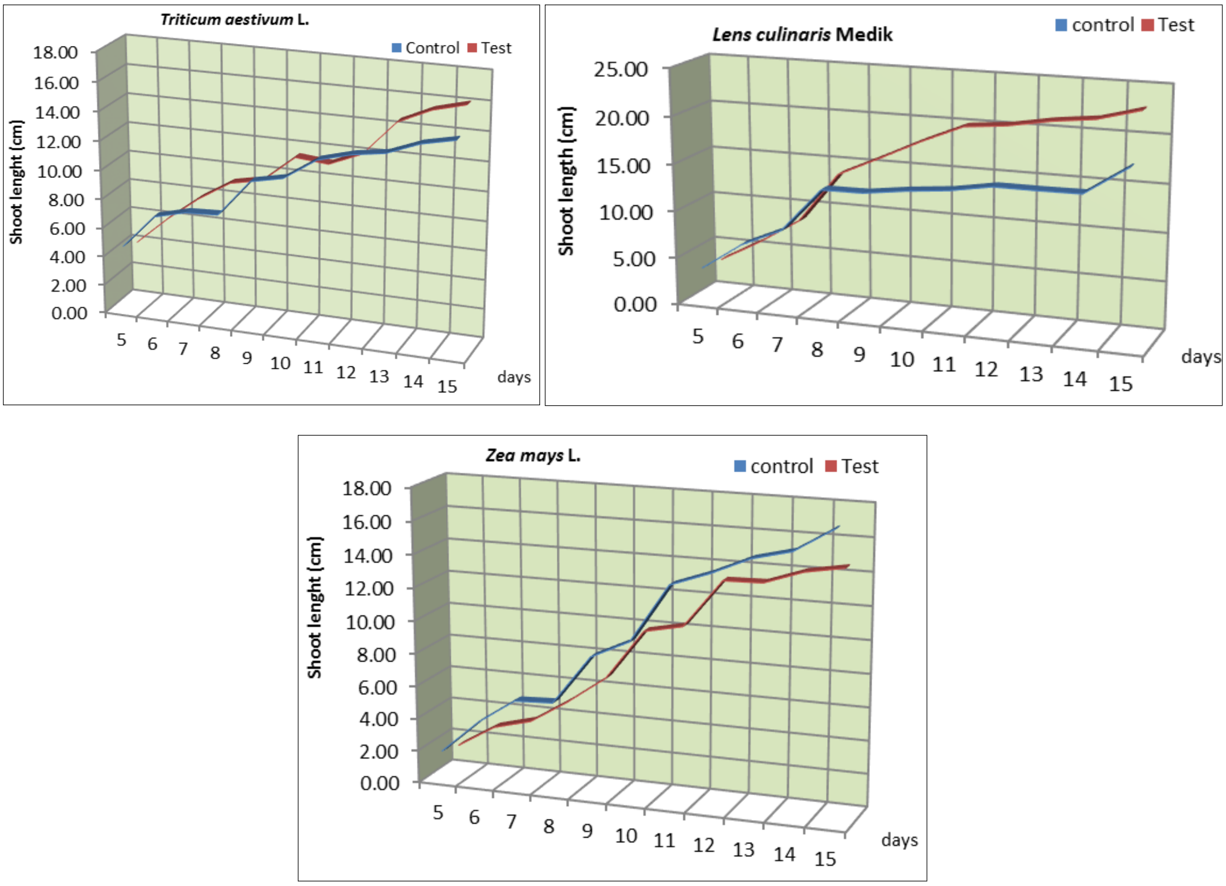

Fig 3: Effect of 0.05% thymol on the length growth of shoots

The biomass, expressed in dry weight (DW) and relative water content (RW) were measured, and data was registered in Table 1.

Table 1: Biomass and water content in control and treated samples, expressed in g per 100g fresh weight (%)

	DW (g/100g)	RW (g/100g)	Proteins (g/100g DW)
Triticum aestivum L. (control)	21,33±0.3033	78,67±0.3033	21.54±1.4551
Triticum aestivum L. (treated)	18,07±0.2067	81,93±0.2067	33.03±2.1865
Lens culinaris Medik. (control)	20,22±0.4500	79,78±0.4500	14.67±0.9007
Lens culinaris Medik. (treated)	17,26±0.3099	82,74±0.3099	23.47±0.5706
Zea mays L. (control)	20,85±0.1700	79,15±0.1700	12.42±0.7625
Zea mays L. (treated)	15,67±0.0301	84,33±0.0301	24.19±1.1886

Exposure of different seedlings to the concentration of 0.05% thymol resulted in a decrease in biomass. The largest decrease in biomass is observed in the corn sample, where the amount of biomass measured is 15.67g dry matter in 100g fresh matter. On the other hand, treated shoots are rich in water and have a high level of total protein.

3.2.3. Photosynthetic pigment content

The quantitative difference of chlorophyll and carotenoids content between controls and treated young shoots were observed (figure 4). Chlorophyll (Ch) is an important pigment to maintain plant growth, composed of Chlorophyll a (Ch a) and Chlorophyll b (Ch b). In all the cases, controls (c) showed higher chlorophyll content in comparison to treated (t). The Chl total content of the three samples; wheat (W), Lentil (L) and Corn (C) was significantly reduced by treatment with thymol (0.05%). Thymol treatment caused a relatively higher decrease of Ch total in wheat, and corn shoots.

Total chlorophyll content decreases from 18.91% to 12.50% in young wheat shoots; which equates to a 33.90% reduction, from 17.70% to 11.36% in corn shoots with a reduction percentage of 35.82%, and for lentils, we observed a small decrease in chlorophyll production compared to the other species aforementioned, a reduction of only 15.80% but this species has the lowest levels of total chlorophyll; 9.98% for controls and 8.40% for treated.

The content of chlorophyll a (Ch a) and b (Ch b) have also been recorded. Chlorophyll a is the majority and more important than chlorophyll b in all three samples also in treated shoots as untreated shoots (controls). In addition, thymol treatments caused much more reduction of Ch a, resulting in decreased Ch a/b ratio, exception of corn sample, which diminution was extremely low; statistically insignificant. The calculated ratio for wheat, lentil, and corn is 2.33; 2.70; 1.99 for controls and 1.21; 1.75; 1.92 for treated.

The figure 4 also shows that the effects of thymol treatment on chlorophyll b content are different from those manifested on chlorophyll a and that the action of thymol on the production of chlorophyll b varies with the plant species. The treatment of young lentil shoots with thymol results in a 34.40% decrease in chlorophyll b production compared to untreated shoots; receiving only water; while wheat shoots have been insensitive to thymol in the synthesis of chlorophyll b. On the other hand, we recorded an increase in the production of chlorophyll b of 12.96% in thymol-treated shoots from corn species.

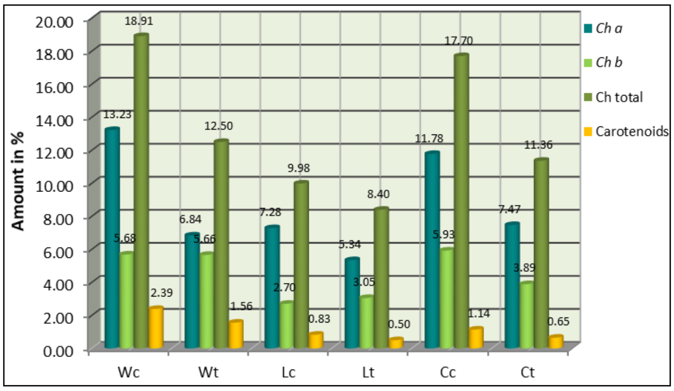

Fig 4: effect of 0.05% thymol on chlorophylls and carotenoids content (c: control, t: treated)

Significant differences were also found in the content of carotenoïds between controls and treated (figure 4), where all three species include low levels of carotenoïds, treatment with thymol solution pronounces the lowering of more than 34%. The comparison of the content of chlorophylls and carotenoïds indicated that the total chlorophyll, chlorophyll a and chlorophyll b content was significantly higher than the carotenoïds content of shoots in all species, with or without treatment.

According to these results, obtained from *in vitro* and *in vivo* tests, the use of thymol as a biopesticide for the protection of crops and seeds must be achieved with greater attention and vigilance. Allelopathy is referring to the negative effects of one species on the germination, growth, or development of other plant species through the release of chemical substances into the immediate environment and the subsequent change of various physiological functions [22]. This phenomenon is observed in nature by scientists and industrialists to develop products based on these chemicals, intended for weeds control. A range of studies have tested these natural products, either in their blend form (essential oils and extracts), or in fractional forms, with a reduced number of chemical compounds, or in pure form where the tested product is composed of a single natural constituent, which has been isolated and purified beforehand, or the pure compound can be a copy of the natural product obtained by chemical synthesis. Scientific research have revealed that monoterpenes can affect the germination of seeds at very low concentrations and as a result, the germination of some weeds species has been strongly inhibited by these monoterpenes [23-25]. If some oxygenated monoterpenes exhibited potent weeds suppressing effects, despite there is no evidence of the phytotoxic effects against various plants of economic interest. In this study, we have just shown that oxygenated monoterpenes, represented by thymol, can affect the germination and growth of other plant species, which are not weeds, and in some are of food and nutritional importance to humans and to the economy, such as wheat, lentils and corn. We have shown that thymol is a low-dose germination inhibitor, and should be used with caution and at very low concentrations in the protection and preservation of seeds against pathogenic germs, in order to suppress its allelopathic effect on seeds. *In vitro*, germination results revealed that the maximum tolerable and non-allelopathic dose is in the order of 0.05%. The treatment of seeds at this dose has no inhibitory effect on germination and the seeds will therefore be able to germinate identically to the control seeds, but *in vivo* growth tests have shown inhibitory effects of this dose on some parameters of growth after seed germination. Negative effects on seedling development are observed mainly on plant biomass, and on the biosynthesis of photosynthetic pigments. The other parameters tested are either not affected or slightly affected or the action is different and depends on the species, in some cases, we have found a stimulatory effect, for example on the growth in length and whose stimulating action differs from one species to another.

4. Conclusion

The application of thymol in agriculture as an alternative to chemical pesticides for seed protection and crops meet the dose not allelopathic. If thymol is effective in protecting seeds and crops, it is important that this biopesticide is well controlled in terms of concentration. Our study has just demonstrated that thymol has no allelopathic activity on the germination of wheat seeds, lentils, and corn at the dose of 0.05% under *in vitro* conditions and also *in vivo*. However, it has been shown that certain growth parameters of the seedlings are influenced by this same non-allelopathic dose. We observed, *in vivo*, that the levels of biomass, total chlorophyll, carotenoids were significantly decreased by thymol. Also, a detailed study on the possible effects on other parameters of growth and physiological functions like respiration and ion uptake should be monitored.

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