

Tomato seeds coated with *Thymus vulgaris* L. essential oil nanoemulsion: Antifungal action and maintenance of physiological quality

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Abstract

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Seed treatment represents an alternative to mitigate the occurrence of Fusarium wilt, induced by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (Fol). Although essential oils (EOs) have demonstrated efficacy in controlling Fol both in vitro and in vivo, their constituents exhibit high volatility, low water dispersibility, and a tendency to degrade due to heat. Incorporating EOs into nanoemulsions or biodegradable coatings represents delivery systems that can help prolong their antifungal efficacy and minimize phytotoxicity in seed treatment. The objective of this research was to evaluate the effect of delivery systems incorporated with *Thymus vulgaris* L. essential oil (TEO) on the physiological quality of tomato seeds infected with Fol. The physiological tests performed on tomato seeds were: moisture content determination, germination test (G), first germination count (FGC), emergence speed index (ESI), accelerated aging test (AA), and image analysis. Seeds of the Ibiza and Napolitano cultivars inoculated with Fol were coated with TEO nanoemulsion (TEON) or biodegradable coating (CTEON) incorporated with TEON at 0, 0.5, or 1.0% (w/w). Treatment with TEON at concentrations ranging from 0 to 1.0% (w/w) did not interfere with the physiological quality of seeds from either cultivar. On the other hand, CTEON acted as a physical barrier, hindering the emission of the primary root and causing delays in the initial germination phase. Seeds treated with CTEON incorporated with TEON showed symptoms of phytotoxicity at concentrations between 0 and 1.0% (w/w). Considering the beneficial effect of TEON on seed health, further studies are needed to establish the ideal conditions for tomato seed treatment.

Keywords: volatile oil; thyme; alternative control; *Fusarium* spp.; phytotoxicity; olericulture

Introduction

Brazil is the eighth largest producer of tomato (*Solanum lycopersicum* L.) in the world, with an average production of

three million tons per year. It is the second most economically significant vegetable crop in the country, second only to potatoes, being marketed fresh or processed (Gueiros et al., 2019).

To ensure proper plant establishment in the field, it is extremely important to use seeds that meet the attributes of genetic, physical, physiological, and sanitary quality (Nóbrega and Nascimento, 2020). Among these, sanitary quality assesses the presence of pathogens in the seeds capable of causing deleterious effects (Zuffo et al., 2023), reducing germination, vigor, and consequently the crop yield (Boregowda et al., 2019).

Among the fungal diseases that affect tomato cultivation, fusariosis wilt stands out, caused by different races of the soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* (Fol). Due to its ability to produce resistant structures, it can remain viable in the soil and crop residues for years, being primarily disseminated through contaminated seeds and soil (Bahadur, 2022).

The use of fungicides is the primary way to control pests and diseases in tomato crops. While fungicide application has brought benefits to the productivity of horticultural crops, the practice poses risks of contamination, pesticide residues in fruits, environmental impacts, increased costs, and the development of resistant strains (Junior, 2019).

In this context, the development of effective and low-toxicity alternatives, in line with the principles of sustainable agriculture, such as the use of natural products derived from plants commonly used as food, can be an option that represents considerable gains for vegetable production (Carmello and Cardoso, 2018).

Essential oils (EOs), compounds derived from the secondary metabolism of plants and biosynthesized by them, have antifungal action and can be used to control phytopathogenic fungi (Goldammer, 2019). Thyme essential oil (TEO), mainly contains thymol and carvacrol which have been confirmed to have antifungal activities against Fol (La Torre et al., 2016; Gonçalves et al., 2021). However, the incorporation of EOs directly presents some important challenges due to high volatility, low solubility and the tendency of their bioactive compounds to degrade when exposed directly to heat (Abouaitah et al., 2023).

The preparation of a delivery system for bioactive compounds, featuring protection of bioactive compounds and controlled release, is a technology that arouses interest for the incorporation of EOs aiming for application in various sectors of the economy (Cao et al., 2023; Gholamhosseinpour et al., 2023; Hammad and El-Sagheer, 2023; Pormohammad et al., 2021; Refaey et al., 2023). Therefore, the objective of this research was to evaluate the effect of delivery systems incorporated with *Thymus vulgaris* L. essential oil (TEO) on the physiological quality of tomato seeds infected with Fol.

Material and Methods

Materials

Thyme essential oil (TEO), (*Thymus vulgaris* L.) (Qui-nari: Lot BJ62640), Tween 80® (Dinâmica Química Contemporânea Ltda: Lot 29126), Cassava starch (Matuto, Lot MT196, Brazil) and Glycerol (Dinâmica Ltda, Lot 65656, Brazil) were commercially acquired. Ibiza and Napolitano tomato seeds were obtained from the genetic improvement program of the crop, from the Vegetable Sector of the Department of Agronomy at the Federal University of Lavras (UFLA). The *Fusarium oxysporum* f. sp. *lycopersici* (Fol) race 3 was obtained from the fungus bank of the Department of Phytopathology (UFLA).

Obtainment of the delivery systems loaded with TEO

The analysis of gas chromatography-mass spectrometry (GC-MS) of TEO identified *p*-cymene (29.99%), carvacrol (21.18%) and thymol (18.17%) as the major chemical constituents. Nanoemulsion was prepared as an aqueous solution composed of TEO and 5% (w/w) Tween 80® in distilled water according to the methodology proposed by Mahdi Jafari et al. (2006) and Tadros et al. (2004). The tubes housing the components underwent agitation utilizing a vortex mixer (MA-162, Marconi, Brazil) for 2 minutes. Subsequently, the emulsions were subjected to ultrasound treatment (QR 750, Eco Sonics, Brazil), aimed at diminishing the droplet size. This ultrasound treatment was conducted within a water bath at ice temperature, employing a titanium microtip (4 mm) for a duration of 4 minutes, operating at a frequency of 20 kHz and an output power of 750 W. To ensure adequate sealing, the emulsions were stored in hermetically sealed test tubes and maintained at room temperature (25 ± 2 °C). The film-forming solutions (FS) forming the coatings were prepared using the casting methodology. Initially, cassava starch and glycerol were added to autoclaved deionized water, and the dispersion was stirred and slowly heated until gelatinization. Then the FS obtained were cooled to room temperature (25 ± 3 °C) to incorporate the TEON (Guedes et al., 2021).

Tomato seeds infection

Initially, a random sample of seeds from each cultivar was disinfected by immersion in a sodium hypochlorite solution (0.25%) for 30s to isolate endophytic fungi. Subsequently, the tomato seeds were infected with the fungus Fol. For this purpose, the fungus was isolated and multiplied through the subculturing process, being inoculated on PDA culture medium and maintained in petri dishes for 7 days at a temperature of 25 °C and a photoperiod of 12h to multiply the inoculum. After the fungus spread across the plates, the

tomato seeds were contaminated by depositing them onto the fungal mycelium, ensuring that all seeds remained in contact with the structures. The incubation period of the seeds on the fungal colonies was 72h (considered the optimal level of infestation in preliminary tests).

Physiological tests performed on tomato seeds

1. Seed treatment

Tomato seeds from cultivars Ibiza and Napolitano infected with Fol were submerged for 10 minutes in 10 mL of: TEON at 0.0% (w/w) (T3 – Tween 80® at 0.5% (w/w) in distilled water), TEON at 0.5% (w/w) (T4), TEON at 1.0% (w/w) (T5), CTEON at 0.0% (w/w) (T6 – cassava starch and glycerol in distilled water), CTEON at 0.5% (w/w) (T7) and CTEON at 1.0% (w/w) (T8). Control 1 and Control 2 were prepared by submerging non-infected (T1) and infected tomato seeds with Fol (T2) for 10 minutes in distilled water, respectively. Afterward, the seeds were air-dried for 24h at room temperature (25 ± 1 °C). After drying, the same procedure was performed again for the tomato seeds from treatments T6, T7, and T8. The repetition was carried out to form the second coating layer.

2. Germination and growth measurements

Moisture content determination: the oven method at 105°C for 24h (Brasil, 2009) was used. Approximately two grams of seeds were placed in each container, with two repetitions for each treatment. The results were expressed as a percentage.

Germination test (G): conducted with 4 repetitions of 50 seeds distributed in germination boxes with blotting paper, moistened with distilled water equivalent to 2.5 times the weight of the dry substrate. These boxes were kept in BOD germination chambers, with a temperature of 25°C and a photoperiod of 6 hours of light and 18h of darkness according to the prescriptions of the Seed Analysis Rules (RAS) (Brasil, 2009). Evaluation took place on the fourteenth day after sowing, determining the percentage of normal seedlings, abnormal seedlings, and dead seeds.

First germination count (FGC): conducted using the same methodology described earlier for the germination test, with the percentage of normal seedlings counted 5 days after sowing (Brasil, 2009).

Emergence speed index (ESI): conducted with four repetitions of 50 seeds for each treatment. The seeds were distributed in plastic trays with a soil and sand mixture in a ratio of 2:1 and placed in a growth chamber with a temperature of 25°C and a photoperiod of 6h of light and 18h of darkness. During the 14 days of the test, the emerged seedlings from the soil were counted daily. The index was

calculated according to the formula proposed by Maguire (1962).

Accelerated aging test (AA): four repetitions of 25 seeds were used, distributed in plastic germination boxes, each containing an aluminum mesh tray inside, where the seeds were spread in a single layer on the surface of the mesh. Inside each box, 40 mL of water were added. The boxes, covered, were kept in BOD germination chambers for an aging period of 72h at a temperature of 41°C. After the aging period, the samples were germinated according to the germination test. Evaluation was conducted five days after sowing, counting the number of normal seedlings (Krzyzanowski et al., 2020).

Image analysis: For measurement of seedling characteristics, 50 seeds from each treatment were germinated following the same methodology as the germination test. Image capture and evaluations took place 14 days after sowing. Mean values of seedling characteristics such as root length (RL), hypocotyl length (HL), and total length (TL) were extracted. The results were expressed in cm/plant. For image capture, the GroundEye® system, version S800, was used. Seedlings were placed in the capture module tray to obtain high-resolution images. In the analysis configuration for background color calibration, the HSV model was chosen with brightness calibration from 0 to 1.00, hue from 95.1 to 317.2, and saturation from 0.459 to 1.00. The fill background was not selected, the minimum object discard size was set to 0.008 cm², and dilation was set to -0.006 cm.

Statistical analysis

The experimental design used was completely randomized (CRD), with 8 treatments and 4 replications for each cultivar. The results were subjected to analysis of variance, and means were compared using the Scott-Knott test at a 5% probability level. To verify the effect of treatments on the variables, analysis of variance (ANOVA) and multivariate statistics through principal component analysis (PCA) were employed. The results were expressed by a two-dimensional plot (biplot). All statistical analyses were performed using the R® statistical software (R Core Team, 2022).

Results and Discussion

For the Ibiza cultivar, when analyzing the variable first germination count (FGC), it is observed that treatments 1 to 6 did not differ from each other (Figure 1A), indicating that this parameter was not efficient in distinguishing different levels of physiological quality. Slow germination is a characteristic of vigor that precedes germination loss. Germination speed is one of the first characteristics to be affected by

seed deterioration (Marcos Filho, 2015). However, the first germination count vigor test indirectly evaluates the seed germination rate (Silva et al., 2019), showing low sensitivity to the reduction of germination speed.

Compared to the other treatments, treatments 7 and 8 showed lower initial germination speed results, with values up to 80% lower when compared to the control (T1).

It is noteworthy that treatments 7 and 8 are the treatments with 2 layers of coating incorporated with thyme essential oil nanoemulsion (TEON). The seed coating agent significantly impacts the duration of imbibition phases and active germination metabolism, as it affects water absorption, necessary for tissue hydration, and the supply of oxygen to the embryo (Javed et al., 2022).

For the Napolitano cultivar, a different behavior was observed (Figure 2A) for the PC variable, where there was a better stratification into quality levels. Treatments 1, 2, and 4 had the highest means, while treatments 3, 5, and 6 had intermediate values, and treatments 7 and 8 had the lowest. These last two were similar to the Ibiza cultivar.

For both cultivars, there is a notable decrease in FGC when the nanoemulsion is combined with two layers of coating. It is known that the coating can act as a physical barrier,

hindering the emergence of the primary root and causing a delay in the initial phase of germination. Additionally, it can restrict gas exchange, which is responsible for supplying the oxygen necessary for the onset of the germination process (Lopes and Nascimento, 2012).

The same occurred in the study by Oliveira et al. (2003), who coated tomato seeds with materials such as microcellulose, limestone + microcellulose, and sand. Upon evaluating their behavior during storage, they concluded that coated seeds exhibited slower germination compared to uncoated ones.

For the variable G (germination at 14 days with the attainment of normal seedlings) for the Ibiza cultivar (Figure 1B), a similar behavior to the FGC variable was observed, where treatments 1 to 6 showed higher means and statistically differed from treatments 7 and 8. The latter, in relation to the control, was the only treatment that exhibited a germination value below the minimum required for the commercialization of tomato seeds, which is 80% and they showed the highest percentage of hard seeds in the germination test. Therefore, it would be the only treatment that would fail the lot for sale as seed (MAPA, 2019). A similar behavior was observed in the Napolitano cultivar (Figure 2B- 2D).

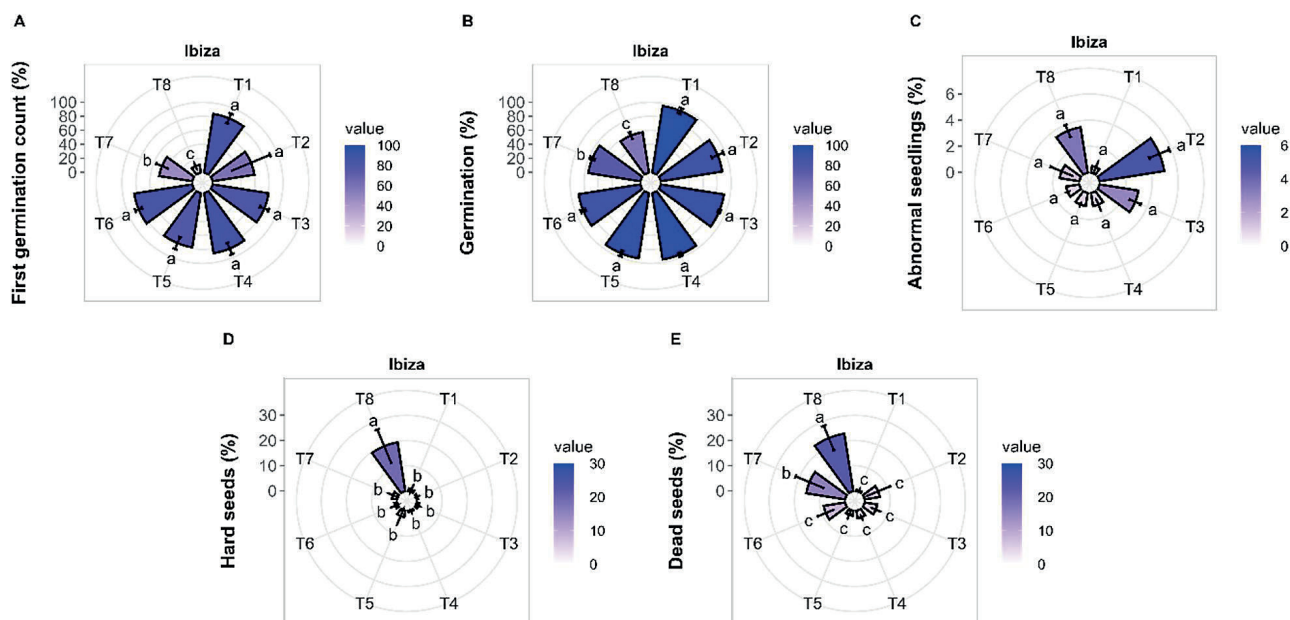


Fig. 1. Average values of first germination count – FGC (%), Germination – G (%), Abnormal seedlings- ANOR (%), Hard seeds – D (%), and Dead seeds – MOR (%) of tomato from the Ibiza cultivar, subjected to different treatments. Means followed by the same letter, within the quadrant, do not differ from each other by the Scott-Knott test at 5% probability

Source: Authors' own elaboration

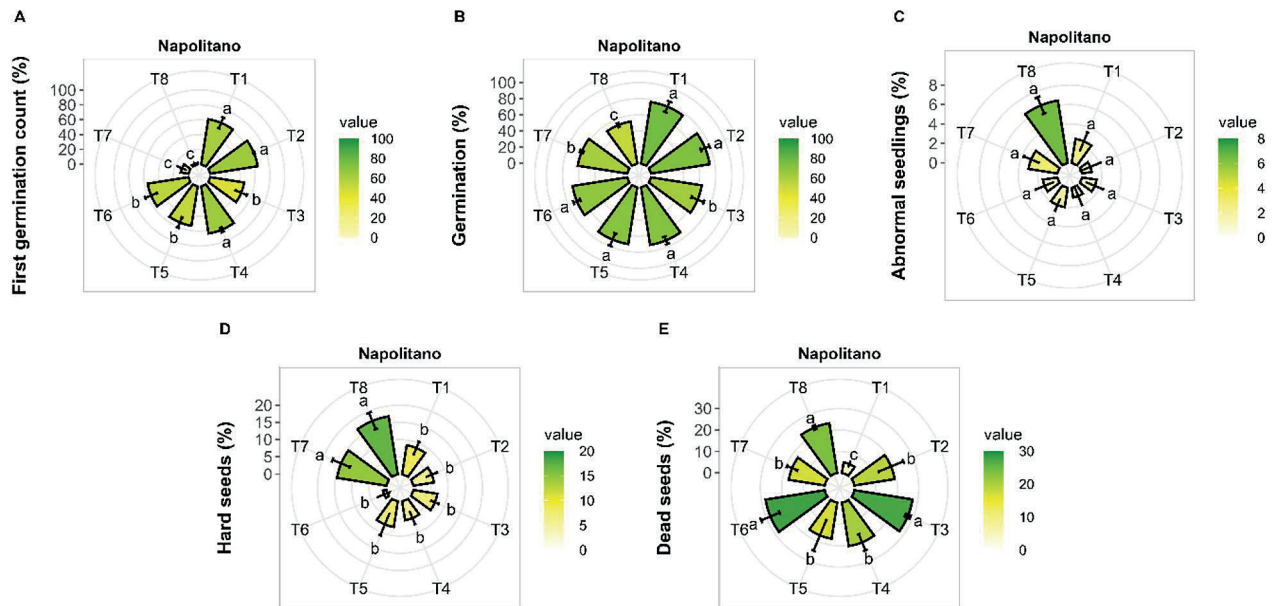


Fig. 2. Average values of first germination count – FGC (%), Germination – G (%), Abnormal seedlings – ANOR (%), Hard seeds – D (%), and Dead seeds – MOR (%) of tomato from the Napolitano cultivar, subjected to different treatments. Means followed by the same letter, within the quadrant, do not differ from each other by the Scott-Knott test at 5% probability

Source: Authors' own elaboration

For both cultivars (Figure 1C-2C), the values observed for the variable abnormal seedlings in all treatments suggest that there was no phytotoxic effect on germination. In a study with lettuce seeds, Miranda et al. (2015) observed that thyme essential oil, when the evaluation of volatile constituents was performed jointly, had no phytotoxic effect on the seeds, where necrosis is the most evident symptom, as well as seedling oxidation.

In the Ibiza cultivar, the quantity of hard seeds was higher in treatment T8, with no hard seeds observed in the other treatments (Figure 1D). However, for the Napolitano cultivar, the highest observed presence of dead seeds occurred in treatments 7 and 8.

The observed value of dead seeds in treatment 8 of the Ibiza cultivar was significant compared to the others (Figure 1E). This suggests that high concentrations of essential oils and the presence of coating layers influenced the mortality of tomato seeds.

For the Napolitano cultivar (Figure 2E), it is noticeable that the quantity of dead seeds in treatment 1, that is, in the control, was lower than in the other treatments. This result highlights that the effect of essential oils varies according to the genotype.

Figures 3 and 4 present the data regarding the IVE for the Ibiza and Napolitano cultivars, respectively.

It is observed that for the Ibiza cultivar (Figure 3A-B), treatment 1 with water only showed the highest index of germination speed as well as a higher cumulative emergence compared to all other treatments, being the most vigorous. The highest speed index of seed emergence without coating was to be expected, as the coating constitutes a physical barrier to root protrusion, thus delaying germination. In this context, a high germination speed indicates high rates of metabolic activities, resulting in high growth rates and early completion of the germination and seedling emergence phase (Krzyzanowski et al., 2020).

Lower values were observed in treatments that presented greater physical barriers of coating and nanoemulsion. For these treatments, the joint analysis of IVE and other physiological quality variables was crucial in observing the reduction in seedling emergence and the increase in the required mean emergence time. Therefore, subjecting the seeds to treatment with TEO (coated or not) affected the IVE of tomato seeds.

The same behavior was not observed for the seeds of the Napolitano cultivar (Figure 4A-B), where treatments did not differ statistically from each other. This reinforces that the

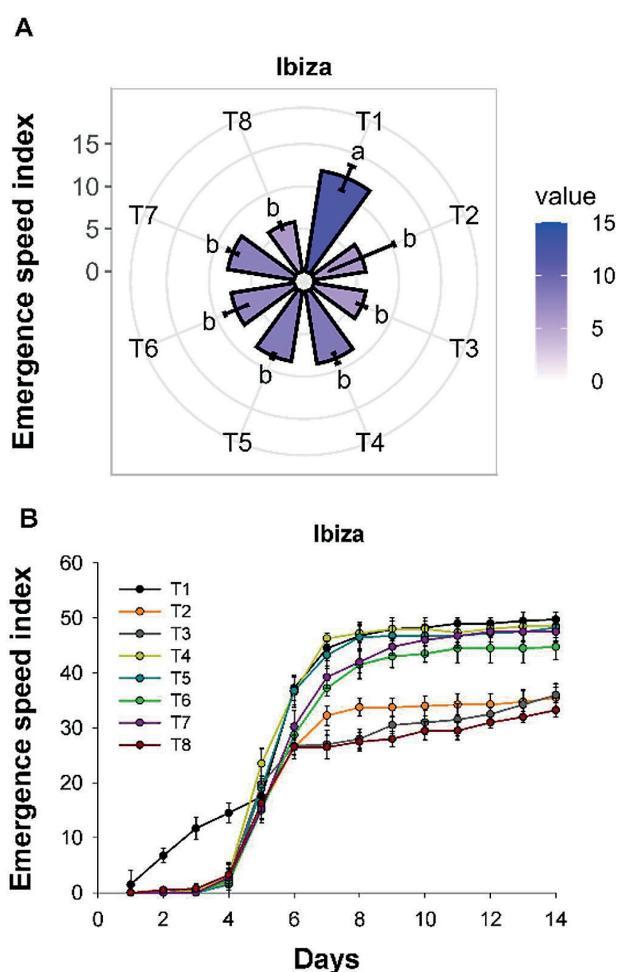


Fig. 3. A – Effect of treatments on the speed of emergence of tomato seeds (cultivar Ibiza). B – Cumulative proportion of tomato seedling emergence (cultivar Ibiza) relative to test days. Means followed by the same letter in the quadrant do not differ significantly according to the Scott-Knott test at 5% probability

Source: Authors' own elaboration

effectiveness of the essential oil depends on the species used, the dosage, and the major compounds present in the oil.

As observed in this study, research conducted by Rosado et al. (2009) demonstrated that the physiological quality of tomato seeds treated with basil essential oil only showed a reduction in the quality parameter of germination speed index (GSI).

An important aspect to evaluate in the present study is the performance of inoculated and treated seeds under adverse conditions. For this purpose, the accelerated aging test (AA) was conducted, which allows the differentiation of

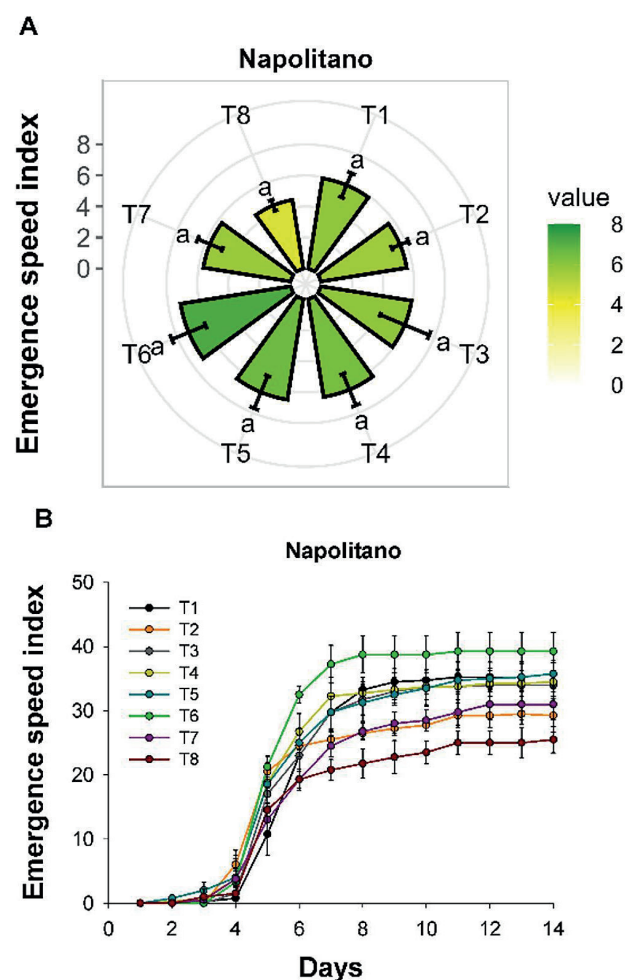


Fig. 4. A – Effect of treatments on the speed of emergence of tomato seeds (Napolitano cultivar). B – Cumulative proportion of tomato seedling emergence (Napolitano cultivar) relative to test days. Means followed by the same letter in the quadrant do not differ significantly according to the Scott-Knott test at 5% probability

Source: Authors' own elaboration

treatments in terms of quality levels when subjected to high temperatures and humidity.

Cultivar Ibiza (Figure 5A) showed higher results in treatments where only fungal inoculation occurred and in treatments where fungal inoculation was combined with thyme EO nanoemulsion, regardless of concentration. These results, combined with previous findings indicating higher physiological quality in these treatments, suggest that when seeds are exposed to the most adverse and favorable deterioration conditions, they survive at high rates. This demonstrates the efficiency of treatments with thyme EO nanoemulsion.

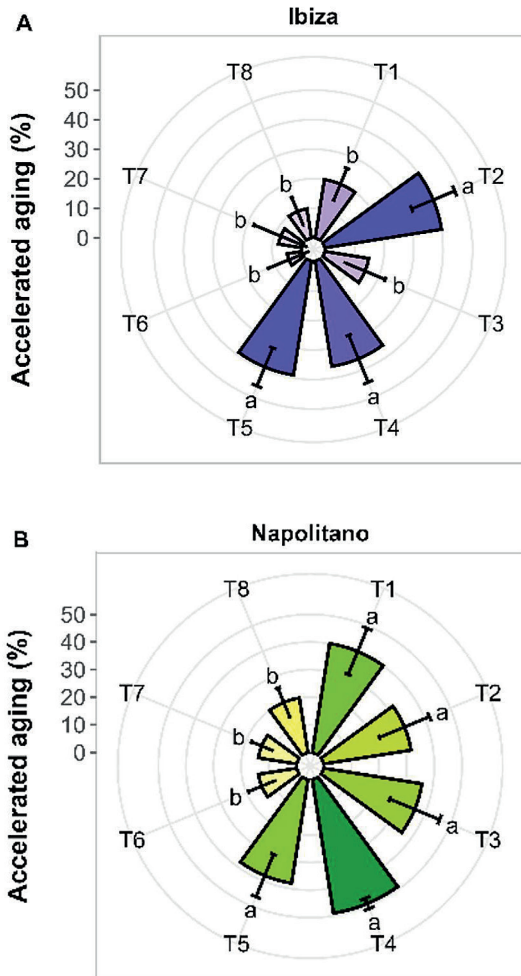


Fig. 5. Evaluation of the different treatments subjected to the accelerated aging test of the Ibiza (5A) and Napolitano (5B) cultivars. Means followed by the same letter, in the quadrant, do not differ from each other by the Scott-Knott test at 5% probability

Source: Authors' own elaboration

For both cultivars, however, it is reinforced that when seeds are treated with two layers of coating regardless of the concentration, the vigor of the seeds decreases drastically compared to the other treatments (Figure 5A–B).

For the variables obtained through image analysis, for the Ibiza cultivar (Figure 6), it was observed, in general, that the development of the aboveground part was greater than the root part. The root length was negatively affected with the increase in nanoemulsions and coating layers. The importance of adequate root development for vegetable crops is

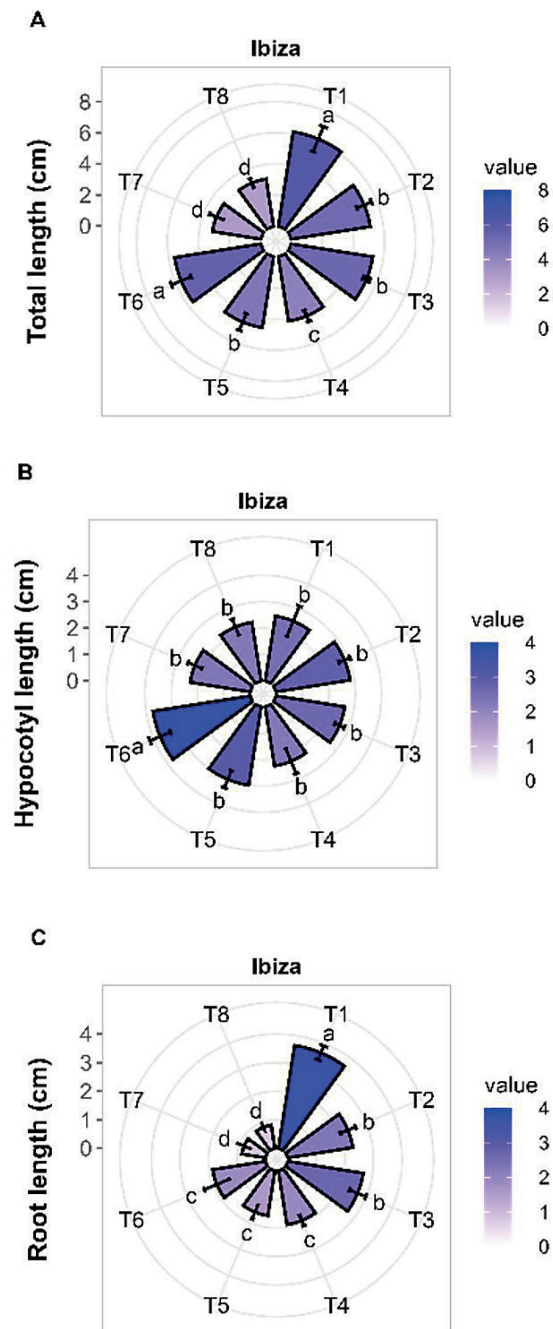


Fig. 6. Total length (A), hypocotyl (B), and primary root (C) characteristics at 14 days of tomato seedlings (Ibiza cultivar). Means followed by the same letter in the quadrant do not differ from each other by the Scott-Knott test at 5% probability

Source: Authors' own elaboration

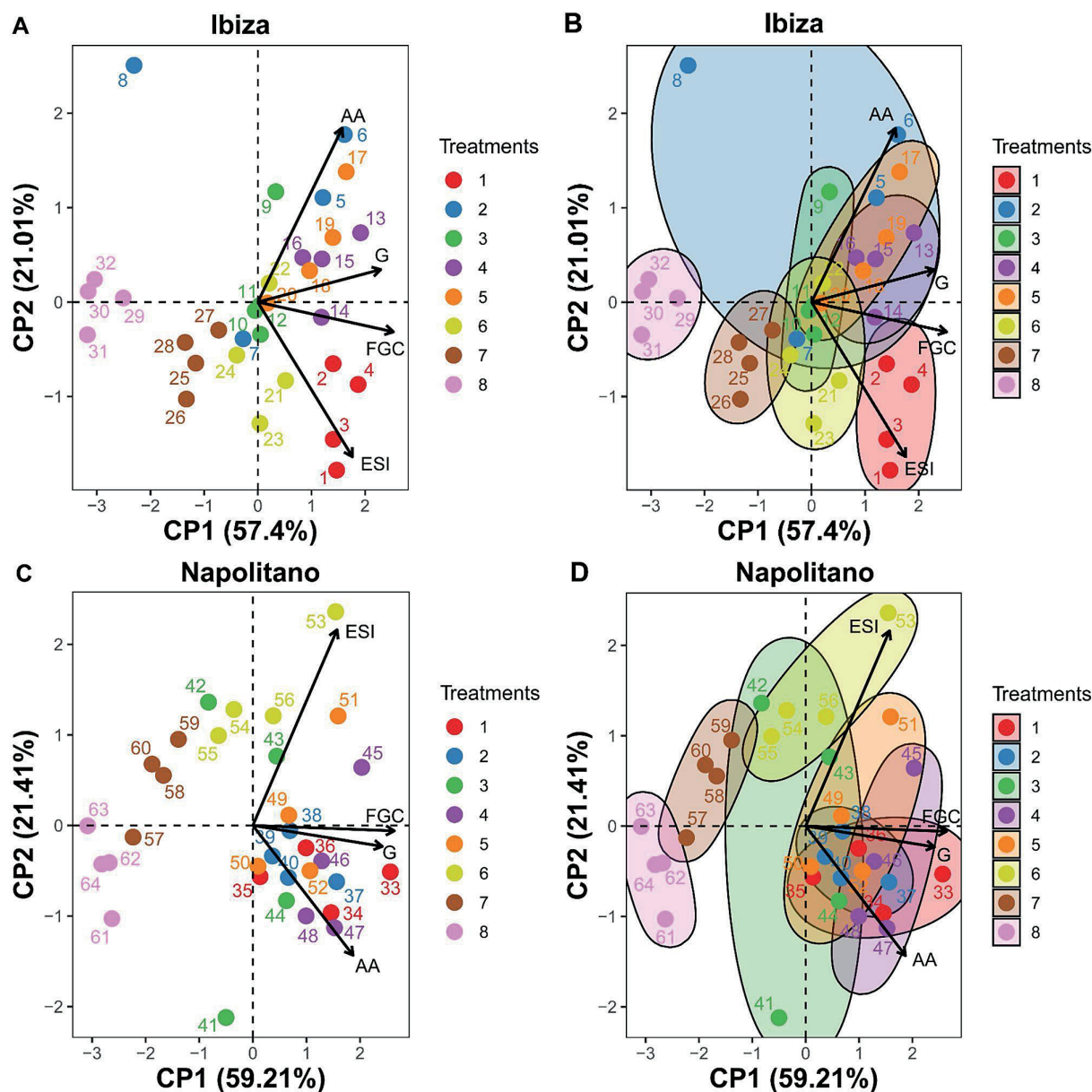


Fig. 7. Biplot of principal component analysis (PCA) considering the treatments highlighting the regions (clustered data) for cultivar Ibiza (A) and (B), and Biplot of principal component analysis (PCA) considering the treatments highlighting the regions (clustered data) for cultivar Napolitano (C) and (D)

Source: Authors' own elaboration

emphasized considering the stresses caused by transplanting seedlings to the field, which directly affects the resumption of root and overall plant development (Vendruscolo et al., 2016).

The lesser development of root organs may be related to the intrinsic growth characteristics of the species under study

or attributed to both differences in the composition of the essential oil constituents, as well as the concentration at which each constituent is found in each oil.

Plants from untreated seeds (control T1) and seeds only inoculated with *F. oxysporum* (T2), in both cultivars, showed a more balanced growth between the shoot and the roots.

In the principal component analysis for both cultivars Ibiza and Napolitano (Figure 7), considering the four variables: first germination count (FGC), accelerated aging (AA), germination (G), and emergence index (IVE), it is reaffirmed that treatments 7 and 8 have their points concentrated in the region opposite to the arrows, indicating that these treatments performed worse compared to the other treatments, which are concentrated in the region of the evaluated variables, meaning they have higher and closer values among them. Thus, the principal component analysis (PCA) reaffirms all the knowledge built in this study.

Conclusions

The incorporation of the active coating (CTEON) promoted a phytotoxic effect. Seeds treated with CTEON incorporated with TEON showed symptoms of phytotoxicity at concentrations between 0 and 1.0% (w/w), acting as a physical barrier. Treatment with TEON at concentrations ranging from 0 to 1.0% (w/w) did not interfere in the physiological quality of seeds from both cultivars considering this treatment beneficial to tomato seeds.

Acknowledgments

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