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Essential oils on the control of stem and ear rot in maize

Óleos essenciais no controle da podridão do colmo e da espiga em milho

Glauco Antonio Teixeira[I*] Eduardo Alves[I] Douglas Carvalho Amaral[I] José da Cruz Machado[I] Fabiano José Perina[I]

ABSTRACT

*Stem and ear rot caused by **Stenocarpella maydis** are responsible for severe losses in maize production. Treatment of seeds with fungicides may induce environmental damage. Hence, this study aimed to evaluate the effects of essential oils extracted from **Cymbopogon winterianus**, **Thymus vulgaris**, **Cymbopogon citratus**, **Corymbia citriodora**, **Cinnamomum zeylanicum**, and **Syzygium aromaticum** on the development of* in vitro ***S. maydis**. In addition, maize seeds were treated with these essential oils to determine their possible mode of action and effects. The oils from **S. aromaticum**, **C. zeylanicum,** and **T. vulgaris** inhibited fungal development at concentrations higher than 0.025%. The oils from **S. aromaticum** and **C. zeylanicum** showed seed germination rates of 89.0% and 84.5%, which were higher than that of the control. The oils from **S. aromaticum** and **C. zeylanicum** reduced the pathogen incidence in the seeds to 39.0% and 28.0%, respectively. Further, these oils as well as that from **T. vulgaris** produced lower reduction of maize stand. Scanning electron microscopy examination revealed that essential oils from **S. aromaticum** and **T. vulgaris** acted directly on the conidia, impeding germination. The findings suggest that the oils from **S. aromaticum**, **C. zeylanicum**, and **T. vulgaris** are potential alternatives for maize seed treatment in the control of **S. maydis**.*

Key words: ***Stenocarpella maydis**, **Zea mays**, alternative control, electron microscopy.*

RESUMO

*A podridão-do-colmo e espiga, causada por **Stenocarpella maydis**, é responsável por graves perdas na produção de milho. O tratamento de sementes com fungicidas pode provocar danos ambientais. Por isso, este estudo objetivou avaliar o efeito de óleos essenciais extraídos de **Cymbopogon winterianus** (citronela), **Thymus vulgaris** (tomilho), **Cymbopogon citratus** (capim-limão), **Corymbia citriodora** (eucalipto), **Cinnamomum zeylanicum** (canela) e **Syzygium aromaticum** (cravo-da-Índia) sobre o desenvolvimento de S. maydis* in vitro*. Além disso, sementes de milho foram tratadas com esses óleos essenciais para determinar seus possíveis modos de ação e efeitos. Os óleos de **S. aromaticum**, **C. zeylanicum** e **T. vulgaris** inibiram o desenvolvimento do fungo nas concentrações maiores que 0,025%. Os óleos de **S. aromaticum** e **C. zeylanicum** mostraram taxas de germinação de sementes de 89,0% e 84,5%, as quais foram maiores que a testemunha. Esses óleos reduziram a incidência do patógeno nas sementes para 39,0% e 28%, respectivamente. Além disso, esses óleos, bem como o óleo de **T. vulgaris**, produziram menor redução do estande de milho. O exame de microscopia eletrônica de varredura revelou que os óleos de **S. aromaticum** and **T. vulgaris** agiram diretamente sobre os conídios, impedindo a germinação. Os resultados deste trabalho sugerem que os óleos de **S. aromaticum**, **C. zeylanicum** e **T. vulgaris** são alternativas potenciais para o tratamento de sementes de milho no controle de **S. maydis**.*

Palavras-chave: ***Stenocarpella maydis**, **Zea mays**, controle alternativo, microscopia eletrônica.*

INTRODUCTION

Stenocarpella maydis (Berk.) Sutton [syn. ***Diplodia maydis*** (Berk.) Sacc.] is the causal agent of one of the main diseases in maize, the stem and ear rots. It is widely distributed in the areas of maize production in Brazil. Besides its effect on productivity, it is one of the major pathogens that affects the quality of seeds and grains of maize. The main symptoms of ***S. maydis*** infection are grain rot, stem and ear rot, and leaf spots (BRESSAN & FIGUEIREDO, 2005). This pathogen shows variable occurrence depending on rainfall regime and temperature and is effectively transmitted via

[I]Departamento de Fitopatologia, Universidade Federal de Lavras (UFLA), 37200-000, Lavras, MG, Brasil. E-mail: glauco@posgrad.ufla.br. *Autor para correspondência.

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seeds. Considerable losses are observed in the field under high humidity conditions, especially when the harvest period is increased (CASA et al., 2006).

Treatment of seeds is the most important measure to control the disease and protect the physiological quality of seeds. Chemical treatment ensures application throughout the surface of the seeds, as well as is economical, considering the low concentrations of active ingredients used (MACHADO et al., 2004). However, extensive use of chemicals in the control of the diseases and pests in agriculture increases environmental and toxicological risks and thus identifying alternative methods becomes necessary. Essential oils extracted from some plants species has shown to have promising economic and ecological advantages. KISHORE et al. (2007) verified that the clove oil and cinnamon oil reduced the pre emergence rotting and the post emergence wilting in peanut seeds ***Aspergilus niger***-infested soil. Also, MEDICE et al. (2007) observed the oils such as ***T. vulgaris*** reduced the severity of soybean rust disease in 30%. The parasitic relationship between ***S. maydis*** and maize leaves may be well understood using the Scanning electron microscope (SEM) analysis, which is a valuable tool to study adhesion, germination, penetration, colonization, and reproduction of pathogens (ALVES et al., 2008). In this way, BRUNELLI et al. (2005) and MEDICE el at. (2007) verified that the essential oils act on the conidia germination and on the appessorium formation as well. Therefore, this study aimed to evaluate the effects of essential oils extracted from ***Cymbopogon winterianus*** (citronella), ***Thymus vulgaris*** (thyme), ***Cymbopogon citratus*** (lemon grass), ***Corymbia citriodora*** (eucalyptus), ***Cinnamomum zeylanicum*** (cinnamon), and ***Syzygium aromaticum*** (India clove) on the development of *in vitro* ***S. maydis*** and on corn seeds inoculated with ***S. maydis***. The ultrastructural aspects of the association of this pathogen with maize leaf tissues were also assessed.

MATERIAL AND METHODS

The experiments were conducted at the Laboratories of Seeds Health and Electron Microscopy and Ultrastructural Analysis (LME), Plant Pathology Department, Federal University of Lavras (UFLA). The essential oils were provided by Brasil Portrait Cosméticos Ltda, Sorocaba, SP, Brazil, register codes (7898202871725, 7898202871749, 7898202871756, 7898202871763, 7898202871787).

The ***S. maydis*** isolate was recovered from maize seeds by using the seed health test, incubation on paper substrate (blotter-test). A single spore was collected and grown in potato dextrose agar (PDA) medium, and then preserved at the UFLA Mycology Collection.

First, the inhibition of conidial germination was assessed using enzyme-linked immunosorbent assay (ELISA) plates and by examining individual conidia under a light microscope. The essential oils from citronella, thyme, lemon grass, eucalyptus, cinnamon, and India clove were used at concentrations of 0.05, 0.2, 0.35, and 0.5%; the control treatment included sterilized distilled water and a fungicide (fludioxonil + metalaxyl; Maxim XL®) was used at the concentration of 0.1%. The experiments were conducted in 8 replicates in a completely randomized design (CRD). In each ELISA plate well, 40 µL each of the oil solution and conidial suspension adjusted to 10^4 conidia·mL^{-1} were added. The plate was incubated at 25°C for 15h under a photoperiod of 12h, and then 20µL of lactophenol was added to each well of the plate. In all, 50 conidia were examined per experimental plot , totaling 400 conidia per treatment.

For the evaluation of mycelial growth, 9mL of autoclaved PDA medium was added to petri dishes, followed by addition of a 1mL pre-mixture of oil and water (10 x) containing 1% Tween20. In the center of each dish, a 6-mm diameter disk containing 7-day-old mycelium developed on PDA was deposited, corresponding to the experimental plot. This assay was also conducted in a CRD; the concentrations of essential oils used in this assay were 0.1, 0.5, 1.0, and 2.0%, along with the control and fungicide Maxim XL® at 0.1%; the experiment was conducted in 4 replicates. The dishes were placed in an incubator at 25°C under a 12-h photoperiod. The negative control consisted of a disk of the fungal mycelium on the medium composed of 9 mL of PDA plus 1mL sterile distilled water plus 1% Tween20, and the positive control included the fungicide. The colony diameters in the petri dishes were measured daily, during 7 days. The results were converted to mycelial growth index (MGI) to be adapted to the formula described by MAGUIRE (1962).

The seeds of the hybrid BX945, susceptible to ***S. maydis***, were inoculated through the osmotic conditioning technique described by MACHADO et al. (2004), with some modifications. For this assay, 0.1% of essential oils of India clove, cinnamon, and thyme were used, as described previously. The treatments consisted of these 3 essential oils, the fungicide at 0.1%, non-inoculated seeds, a control containing only restrictor hydric (mannitol), and another control representing the original seed lot, totaling 7 treatments with 4 replicates.

After inoculation, seeds were subdivided into 7 samples, each with 200 seeds. The samples were immersed separately for 5min in solutions corresponding to the respective treatments and dried on sterilized filter paper at room temperature. Dried seeds were submitted to the health blotter method for 14 days as described by BRASIL (2009), and then examined under a stereomicroscope for the determination of ***S. maydis*** incidence. Seed germination was evaluated using the standard method described in the Rules for Seed Analysis in Brazil (BRASIL, 2009).

For the evaluation of seed vigor, treated and non-treated seeds were submitted to standard emergence method and cold test. For the standard emergence test, the seeds were first disinfested with 1.0% sodium hypochlorite solution for 2min and then treated as previously described. The seeds were sowed in plastic trays containing 5L of the substrate Plantmax® and sand (2:1), and both were autoclaved earlier. The numbers of emerged seedlings were counted daily, until emergence stabilization. The emergence seed index (ESI) was calculated using the formula suggested by MAGUIRE (1962). Next, the initial and final plant populations (stands) were counted at 10 and 28 days after sowing.

For the cold test, trays containing 5L of substrate consisting of soil and sand mixture (2:1) were used. The soil was collected from an area previously cultivated with maize. The water supply was as adjusted to the reference level of 70% of the substrate retention capacity. The trays were placed in a cold chamber at 10°C in the dark for 7 days. Subsequently, the trays were transferred to a growth chamber at 25°C with a 12-h photoperiod. The numbers of emerged seedlings were counted after 5 days.

Another experiment was conducted using health maize leaves obtained from plants cultivated in a greenhouse. Two 20-cm pieces of leaves were placed in a plastic tray containing filter paper moistened with sterilized distilled water and covered with aluminum foil. Next, 40 µL of the pre-mixed suspension adjusted to 7.5×10^4 spores·mL^{-1} was added to the solution of each treatment. The treatments consisted of oil solutions from clove, cinnamon, and thyme; the fungicide Maxim XL® at the concentration of 0.1%; and positive and negative controls. The suspension was applied in pre-defined places on the lower surface of the leaves such that samples could be collected with fragments of approximately 0.5cm. These fragments were collected at 3, 7, 11, 15, 20, 25, 30, 35, 40, and 45h after inoculation. The collected fragments were fixed in modified Karnovsky's solution, and then treated as described by ALVES et al. (2008). The number of germinated conidia were also counted by selecting a specific area on the leaf surface; the data were transformed to percentage rate.

The data were analyzed using analysis of variance by using the software R Core Development Team (VENABLES & SMITH, 2010), and the treatment results were compared using Scott–Knott test ($P<0.05$).

RESULTS

Conidia germination was reduced by all treatments except for 0.05% citronella oil, which enhanced conidial germination. Furthermore, eucalyptus oil and fungicide treatment produced an intermediate effect on conidial germination compared to the control (Figure 1).

Mycelial growth of ***S. maydis*** and maize seed emergence did not vary significantly between 1.0% citronella, 0.1% lemongrass, and 0.1% and 0.5% eucalyptus oil extracts and control. On the other hand, thyme and India clove oil extracts and the fungicide completely inhibited mycelial growth of the fungus. Similarly, 0.5% and higher concentrations of cinnamon and lemongrass oil extracts and 2.0% and higher concentrations of eucalyptus oil showed higher mycelial growth inhibition. In contrast, citronella oil stimulated the mycelial growth of the fungus (Table 1).

S. maydis was not found in non-inoculated seeds in these experiments. However, the fungus was found in small numbers in the cinnamon, followed by India clove oil-treated groups, although the latter did not differ significantly from the fungicide-treatment group. The positive control and thyme oil-treated groups showed significantly higher numbers of the fungi compared to all other treatment groups. The emergence and cold tests did not yield significantly different results among the treatment groups, except for seeds treated with mannitol, which showed significantly lower emergence rate than that in the other treatment groups in the cold test (Table 1). ***S. maydis*** produced 21% reduction in seed germination. On the other hand, treatment with all essential oils led to a lower rate of stand reduction (final stand - initial stand), followed by that in the fungicide-treated group and the positive control. However, the control group with mannitol and the negative control group produced a lower reduction of stand index (Table 1).

In non-treated maize leaves, conidia germination was not observed up to 3h after

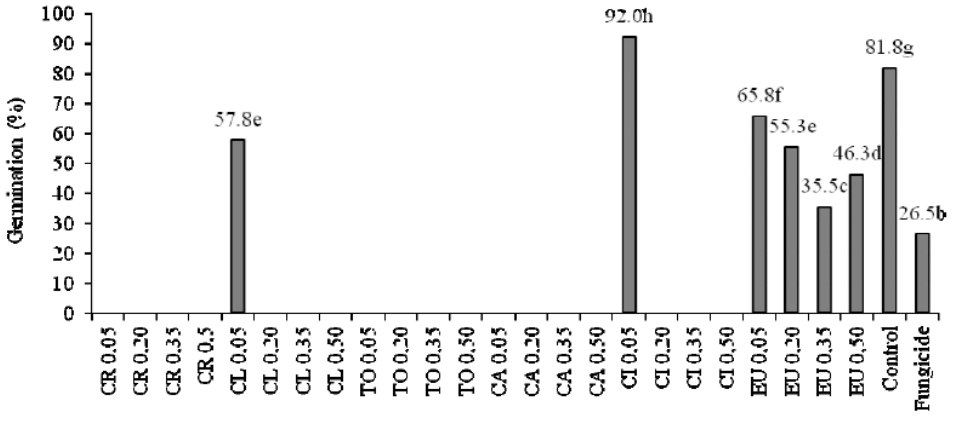

Figure 1 - Effect of essential oils on the percentage of conidial germination of ***Stenocarpella maydis*** in water. TO: thyme; CR: India clove; CA: cinnamon; CI: citronella; EU: eucalyptus; CL: lemongrass.

inoculation (h.a.i.; Figure 2A). Conidial germination started from 7h.a.i. (Figure 2B) and was more pronounced between 11 and 15h.a.i, when 87.5% germinated conidia were observed (Figure 2C). The onset of appressorium formation occurred between 11 and 20h.a.i. and conidial penetration began at 20h.a.i. (Figure 2D and 2F). At 25 h.a.i, 92% of the conidia had been germinated and 50% had appressoria. From 35h.a.i., elongation of the germ tube and development of the penetration structures of the fungus were observed (Figure 2E).

In leaves treated with cinnamon, India clove, and thyme oil extracts, conidial germination began between 11 and 15h.a.i. In the leaves treated with cinnamon and thyme, appressorium formation occurred between 15 and 25h.a.i., and elongation of the germ tube started from 20h.a.i (Figure 2G). However, for leaves treated with India clove oil, appressorium formation was observed at 25h.a.i., with no elongation of the germ tube (Figure 2H). The leaves treated with India clove and thyme oils showed wilting of some of the germ tubes and penetration structures at 30h.a.i (Figure 2I). In the leaves treated with the fungicide, germination was observed only in a few conidia starting at 30 h.a.i., and appressorium formation and elongation of the germ tube were not observed up to 45h.a.i. (Figure 2J).

DISCUSSION

The control of several pathogenic fungi in maize has been successful achieved by treating seeds with chemical fungicides. Depending on factors such as seed physiological quality and inoculum potential of the pathogens in the seed lot, the efficacy of such treatment may vary. With the increase of maize cultivated area in Brazil, the volume seed lots subjected to fungicide treatment also increases, causing a concern from the pollution point of view. In this study, the potential of essential oils extracted from some plant species such as India clove, thyme, and cinnamon used as alternatives for the control of pathogenic fungi was assessed. In the bioassay, all oils completely inhibited mycelial growth and germination of ***S. maydis*** at concentrations higher than 0.5%. Similar results has been obtained by other studies that used garlic extracts (***Allium sativum***) and lemongrass (***Cymbopogon citratus***) at an initial concentration of 0.5% to study the effect on mycelial growth and germination of ***Fusarium proliferatum*** conidia (SOUZA et al., 2007). Similarly, essential oils from sage, garlic, and cinnamon were found to inhibit mycelial growth of ***Aspergillus flavus*** isolates (VIEGAS et al., 2005). LIMA et al. (2006) indicated that the oils from cinnamon (***Cinnamomun zeylanicum***) and Chilean boldo (***Peumus boldus***) inhibited the growth of 58% of ***Candida*** species strains.

Regarding the effect on the quality of maize seeds, ***S. maydis*** was responsible for 21% reduction in seed germination, and none of the treatments was able to eliminate the fungus from infected seeds. Nonetheless, treatment of seeds with the essential oil from cinnamon at 0.1% dose reduced ***S. maydis*** incidence by 49%. Similar reduction in fungal incidence has been reported for other cases such as ***Fusarium proliferatum*** following treatment of maize seeds with garlic extract (***Allium sativum***)

Table 1 - Mycelial growth index (MGI) of ***Stenocarpella maydis***; seed germination and vigor and incidence of ***S. maydis*** in maize seeds treated with essential oils; plant stand size at 10 and 28 days after sowing .

Essential oils	Concentration (%)			
	0.1	0.5	1.0	2.0
India Clove	0.00 a	0.00 a	0.00 a	0.00 a
Thyme	0.00 a	0.00 a	0.00 a	0.00 a
Cinnamon	12.3 b	0.00 a	0.00 a	0.00 a
Lemongrass	14.1 c	0.00 a	0.00 a	0.00 a
Eucalyptus	14.8 c	15.1 b	11.6 b	0.00 a
Citronella	24.6 d	20.4 c	13.9 c	1.4 a
Control	14.81 c		Fungicide	0.00 a

Treatment	Germination of seeds (%)	Incidence of ***S. maydis*** (%)	Cold test (%)	ESI[1]
India clove	89.0 c	39.0 c	62.5 b	24.0 a
Cinnamon	84.5 b	28.0 b	61.5 b	23.7 a
Thyme	77.5 a	55.5 d	67.0 b	23.4 a
Fungicide	84.5 b	40.0 c	72.5 b	24.7 a
TI	76.5 a	57.0 d	69.5 b	25.2 a
TM	92.0 c	0.0 a	40.5 a	25.1 a
TNI	93.0 c	0.0 a	78.0 b	26.4 a
CV (%)	3.51	20.19	12.08	7.87

Treatment	Initial stand (%)	Final stand (%)	Stand reduction (%)
India Clove	78.5 a	71.5 a	8.92 b
Cinnamon	77.0 a	68.0 a	11.69 b
Thyme	76.5 a	67.0 a	12.42 b
Fungicide	81.5 a	67.5 a	17.18 c
TI	82.0 a	62.0 a	24.39 d
TM	84.5 a	81.0 b	4.14 a
TNI	92.0 b	87.5 b	4.89 a
CV (%)	5,60	7,76	33,34

Means followed by the same letter do not differ significantly from the values presented in that column by Scott–Knott test ($P<0.05$).
[1]Emergence seed index.
(TI) inoculated control, (TM) control only with mannitol, and (TNI) non-inoculated control.

and lemongrass (***Cymbopogon citratus***) (SOUZA et al., 2007). This led to an increase of 24% in the germination percentage of the seeds.

By SEM, appressorium formation was observed after 72h of incubation at 25°C. However, BENSCH & STADEN (1992) showed that the germination of ***S. maydis*** conidia in the stems and corn leaves occurred after 5h of incubation at 30°C. In addition, seeds treated with the essential oils from India clove, cinnamon, and thyme delayed the germination of ***S. maydis*** conidia by about 5h. However, for leaves treated with India clove oil, appressorium formation occurred at 25h after inoculation. In the same way, BRUNELLI et al. (2005) conducted SEM analysis and found that 86% of the conidia of ***Stenocarpella macrospora*** germinated between 12 and 15h after inoculation and appressorium formation occurred at 18h.a.i. Furthermore, oils from India clove and thyme caused wilting of some conidia at 30h.a.i.; this finding is similar to that reported by MEDICE et al. (2007) who evaluated the effect of essential oils applied on soybean leaves surface on spore germination of ***Phakopsora pachyrhizi***.

The results suggest that treatment of maize seeds with essential oils from India clove, cinnamon, and thyme, at the concentration of 0.1%, mighty play an important role in the control of stem and ear rot fungus. Besides having a direct effect on the development of ***S. maydis*** *in vitro* colonies these oils suppressed the proliferation of the fungus in maize plant tissues. The use of these oils may contribute to higher maize seed quality ensuring a desired plant population in the field, without causing any risk to man or the environment.

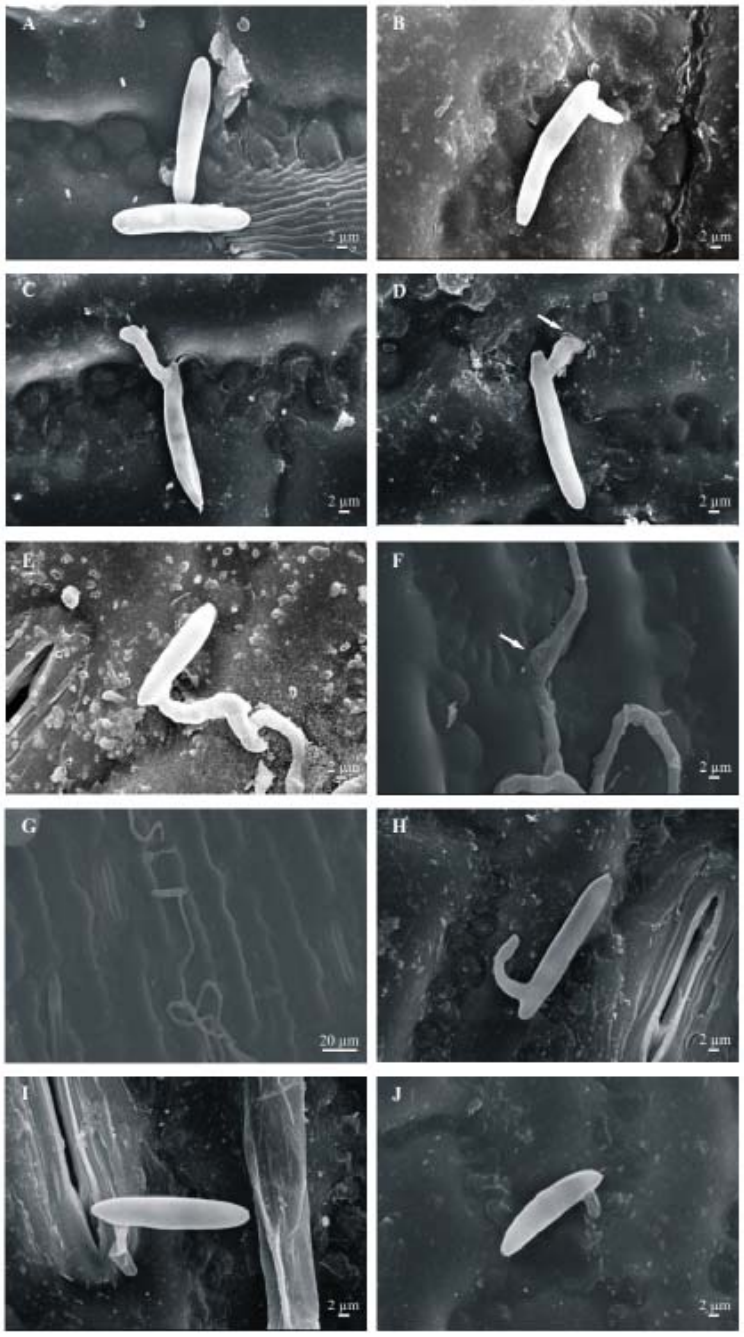

Figure 2 - Scanning electron micrographs of corn leaves inoculated with ***Stenocarpella maydis*** (A–F) and treated with essential oils (G–J). Conidia are not germinated (3 h.a.i.) (A), Conidium at the beginning of germination (7 h.a.i.) (B), Initial formation of appressorium (11h.a.i.) (C), Appressorium formed (20h.a.i.) (D), Germ tube elongation (30h.a.i.) (E), Penetration of the fungus by appressoria (20h.a.i.) (F), Elongation of the germ tube and penetration by appressorium in leaves treated with essential oil from cinnamon (20h.a.i.) (G), Appressorium formation in leaves treated with essential oil from India clove (25h.a.i.) (H), Wilted germ tube in leaves treated with essential oils from thyme and India clove (30h.a.i.) (I), Beginning of conidial germination in a leaf treated with fungicide (40h.a.i.) (J).

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