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Characterization of *Alternaria brassicicola* isolated from tomato in Burkina Faso, and use of two essential oils for its control *in vitro*

Adama SIRIMA[1]*, Abdoulaye SEREME[2], Kadidia KOÏTA[1], Elisabeth ZIDA[3], Abel T. NANA[1], Drissa SEREME[4], Théodore NANA[2] and Mahamadou SAWADOGO[1]

[1]Biosciences Laboratory, UFR/SVT, Joseph KI-ZERBO University, Ouagadougou, 03 BP 7021 Burkina Faso.
[2]Natural Substances Department / IRSAT / CNRST; 03 BP 7047 Ouagadougou 03, Burkina Faso.
[3]Plant Pathology Laboratory, Institute of Environment and Agricultural Research, Kamboinsin Station, 01 BP 476 Ouagadougou 01 Burkina Faso.
[4]Laboratory of Virology and Plant Biotechnology, Institute of Environment and Agricultural Research, Kamboinsin Station, 01 BP 476 Ouagadougou 01 Burkina Faso.

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Alternaria brassicicola is considered a weak pathogen of tomato in the field, but with a high potential for mycotoxin production in fruit. However, this species belongs to a diverse and closely related group of species (_Alternaria_ with small spores), which often makes their morphological identification and control difficult. In the present research, a molecular identification of tomato _Alternaria_ isolates was made using sequence data of the internal transcribed spacer (ITS) region and the 5.8S ribosomal DNA (rDNA) gene of the isolates. Isolates were identified as _Alternaria brassicicola_. They were obtained from samples collected from symptomatic tomato leaves in Loumbila, a locality in the Central Plateau region of Burkina Faso. _In vitro_ antifungal tests with essential oils of _Cymbopogon nardus_ and _Eucalyptus camaldulensis_ showed an inhibitory effect of these oils on _A. brassicicola_. The inhibition rates recorded by the two essential oils at appropriate doses (1, 5, 10, 50 and 100%) are higher than those obtained with the chemical fungicides Azoxystrobin and Mancozeb. The use of formulations based on these essential oils could be an alternative to hazardous pesticide treatments, thus allowing a safer and more ecological management of field diseases and toxins of _Alternaria_ sp. in tomato fruits.

Key words: *Alternaria brassicicola*, molecular identification, biological fungicide.

INTRODUCTION

Fungi of the genus *Alternaria* are a diverse group of organisms (saprophytic, pathogenic), widespread in nature and much studied. Pathogenic species of this large genus usually cause deterioration of field crops or post-harvest products resulting in considerable losses due to fruit rot (Matrood and Rhouma, 2021; Ostry,

*Corresponding author. E-mail: almamybiko@gmail.com.

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2008). They have been shown to have a devastating effect mainly on tomato and also on cucurbits, and brassicas (Mamgain et al., 2013). They are ubiquitous, and are often responsible for the deterioration of products during transportation and storage (Ostry, 2008).

Alternaria brassicicola causes more yield losses in brassicas including cabbage. The disease manifests as leaf spots in the field (Munir et al., 2020; Nowakowska et al., 2016). In contrast, the pathogen is considered occasional or weak and can establish itself late on plants of Resedaceae and Solanaceae, on drying latex of injured trunks of Hevea sp., or in the soil (Lawrence et al., 2015). The development of *A. brassicicola* is influenced by several environmental factors: the optimal temperature for its growth is 25°C, whereas a temperature of 35°C completely inhibits its development (Kiran et al., 2018). Also, the growth and sporulation of the parasite are strongly affected by high light intensities (> 20 lux) (Kiran et al., 2018). Its conidial chains of are moderately long, simple or branched. Mature conidia are ellipsoidal, ovoid, yellowish to dark brown. They are septate, usually with apical only and rarely with lateral secondary conidiospore (Lawrence et al., 2015). They are capable of producing mycotoxins during germination. Indeed, the genus *Alternaria* is one of the main groups of mycotoxigenic fungi reported with more than 70 metabolites (including mycotoxins) that can affect human health (Meena and Samal, 2019; Siciliano et al., 2017). These mycotoxins are alternariol, alternariol monomethyl ether, alterenene, altertoxins I, II, III, tenazonic acid and other less toxic metabolites (Meena and Samal, 2019; Ostry, 2008; Escrivá et al., 2017). They can induce in several animal species, mutagenesis, carcinogenesis, degradation of DNA integrity, disruption of sphingolipid metabolism, inhibition of enzyme activity, etc. (Escrivá et al., 2017; Ostry, 2008). Escrivá et al. (2017), found in tomatoes, oil seeds, carrots, etc., the highest levels of alternariol and alternariol methyl ether. Because of the mycotoxigenic activity of *Alternaria* species, it is necessary to examine their presence on tomato in Burkina Faso, even if the species does not have a significant effect on tomato yield. Also, although each species of the genus has a distinct conidial morphology, it is not surprising that misidentification and confusion very often occurs during their phenotypic identification due to the similarity of some species (Konstantinova et al., 2002). For these reasons, it is necessary to combine the Polymerase Chain Reaction (PCR) test based on the use of developed specific primers and phylogenetic analysis, with the traditional method of phenotypic identification.

Considering the diversity, abundance and important damage caused by species of the genus *Alternaria*, the main method of control of *Alternaria* disease and associated mycotoxins, in many tropical countries, is the use of synthetic chemical fungicides (Mugao et al., 2020), which are harmful to human and animal health, and to the environment. Also, nowadays, consumers are more and more demanding about the quality of vegetable products, which must be free of pesticide residues, microbial growth, toxins, etc. (Hadizadeh et al., 2009). In Burkina Faso, no in-depth study of the pathogens has been carried out to date, to propose an innovative alternative control method to chemical control. It is in this perspective that the present study sets as objectives, a morphological and molecular characterization of *A. brassicicola* isolates isolated from tomato leaves of Burkina Faso using specific primers, and the study *in vitro* of fungicidal effects of essential oils of two aromatic species (*Eucalyptus camaldulensis* and *Cymbopogon nardus*) on the pathogen.

MATERIALS AND METHODS

Plant material

The essential oils of *E. camaldulensis* and *C. nardus* used for the antifungal tests were extracted in the laboratory of the Department of Natural Substances of the Institute of Research in Applied Sciences and Technologies (DSN/IRSAT), located in the industrial zone of Kossodo in Ouagadougou.

Fungal material

The *A. brassicicola* isolate used in this study was obtained from diseased tomato leaves collected from growers' fields in Loumbila (30P 0675454; UTM 1380456; 285 m; S 0 m) in the Central Plateau. Incubation and morphological identification of the isolated species were done in the phytopathology laboratory of the Institute of Environment and Agricultural Research of Burkina Faso (INERA/Farako-Ba).

Isolation and morphological identification of *A. brassicicola*

Infected tomato leaves were washed with sterile distilled water and then cut into small circular pieces about 2 cm in diameter. They were then immersed in 70° alcohol for two minutes. They were then rinsed twice in sterile distilled water and dried on filter paper before being placed in 90 mm diameter Petri dishes containing sterile blotting paper. Petri dishes were incubated at 22°C in an incubation chamber under a 12 h UV light/12 h dark regime for 96 h. A pure culture of the isolate was obtained by transferring conidia obtained on Potato Dextrose Agar (PDA) culture medium. The isolated pathogen was identified as *A. brassicicola* on the basis of conidial morphology using the identification manual of Mathur and Olga (2003). The Gildan Homme 5000 color chart was also used to determine the color of colonies on PDA and Prostate-specific antigen (PSA) substrates. Morphological characteristics such as radial mycelial growth, colony shape and texture, and conidial size were noted.

DNA extraction

A. brassicicola isolate was first grown on PDA medium and incubated at laboratory room temperature for 10 days. Twenty milligram fresh weight of mycelium from each isolate was collected by scraping the surface of the colony and placed in a 2 ml eppendorf tube with 2 beads. Mycelia were ground by mechanical agitation using the Tissue Lyser II grinder (QIAGEN). DNA was

extracted according to the method of Permingeat et al. (1987). The recovered DNA pellet was dissolved in 30 µl of sterile water and stored at -20°C for later use.

Detection by PCR and sequencing

Detection of isolates was done using the oligonucleotide primer pair AAF2/AAR3 according to the method used by authors (Konstantinova et al., 2002; Nabahat, 2014). The AAF2/AAR3 primer pair amplifies a 341 bp fragment corresponding to part of the ITS regions and the 5.8S gene. The amplification products were visualized in a 1% agarose gel using a gel reader, MS UVDI 129-0323, in the presence of a 1000 bp molecular weight marker from the company Promega. PCR products were sequenced directly. Sequencing was performed by Macrogen-Europe, Amterdam (The Netherlands; www.macrogen.com).

Molecular identification

The Blast program of the NCBI website allowed us to compare the sequences obtained after sequencing with an online database (http:// www.ncbi.nlm.nih.gov). Blast provided us with a list of species with high sequence similarity and homology to the one tested.

Extraction of essential oils

The extraction of essential oils was carried out in the laboratory of the Natural Substances Department of the Institute of Research in Applied Sciences and Technologies (DSN/IRSAT), CNRST/Burkina. The essential oils of *E. camaldulensis* and *C. nardus* were extracted from leafy twigs of the plant using a steam distillation with a still.

In vitro antifungal tests of essential oils

The essential oils were diluted at different doses with dimethyl sulfoxide (DMSO) according to Sirima et al. (2020) method. In 100 ml of freshly prepared PDA medium, 2 ml of each dose of essential oil is added (at the temperature of 37°C of the medium). Two synthetic fungicides registered in the member countries of Inter-State Committee for Drought Control in the Sahel (CILSS), Mancozeb and Azoxystrobin, were used as positive controls; and PDA alone was used as a negative control. A mycelial explant of the isolate, measuring 1 cm in diameter, was taken from a pure culture and placed in the center of each Petri dish containing the culture medium to be tested. The tested doses of essential oils were: 1, 5, 10, 50 and 100%. Colony diameters were measured every day until the mycelial growth of the untreated control reached the edges of the bottom of the dish. Comparison of the dimensions obtained with those of the control allowed calculation of the percentage of inhibition according to the formula:

$$\mathrm{Ti} = \left(\frac{\mathrm{D}_{control} - \mathrm{D}_{test}}{\mathrm{D}_{control}}\right) \times 100$$

T_i = % inhibition of radial mycelial growth for one dose of essential oil
$D_{control}$ = colony diameter of the fungus in the untreated control
D_{test} = colony diameter of the fungus for one dose of the essential oil

Data analysis

The results were analyzed by the variance method (ANOVA) using XLSTAT 2016 software. The comparison of the activities of the essential oils between the different concentrations and the fungicide was performed by Duncan's Test at the 5% threshold.

RESULTS

Isolation and morphological characterization

Isolates of *A. brassicicola* obtained from symptomatic tomato leaves collected from tomato produced in Loumbila had the same morphological characteristics. They showed circular colonies on PDA and PSA, without pigment production, but with different staining. On PDA medium, the colonies were ash-grey in aerial view of the culture on the 6th day after incubation; with white margins and a cottony texture on the 4th and 6th day after incubation. The concentric circles, absent until the 4th day, appeared at the 6th day after incubation (Figure 1 (a and e)). On the underside (reverse side of the Petri dish) of the same medium, the colonies had a black color in the center with a hazy yellow rim and white margins (Figure 1b). On the other hand, on the PSA medium, the colonies showed concentric circles, a grey sport coloration on the upper side and whitish peripheries from the 4th day after incubation (Figure 1 (c and f)). On the bottom side of the same Petri dish, the colonies had a black color and whitish peripheries (Figure 1d). In addition, the coloration of the colonies changed with age. The isolate showed quite rapid growth, covering the bottom of the 8.5 mm Petri dish in ten days on PSA medium and in twelve days on PDA medium. Conidia were polymorphic with average sizes ranging from 1.56 to 1.83 µm (Figure 2a, b, c and d). They were gold in color and had transverse and longitudinal septa (Figure 2e).

Molecular identification

PCR amplification resulted in a band of the expected size (341 bp). The amplification band was quite strong for all isolates and with an absence of spurious bands. Sequencing provided the complete 5.8S gene sequence of the ITS region of the Burkina Faso isolates. BLAST analysis, performed by the sequencing company, confirmed that the isolates belong to the genus *Alternaria*. Indeed, the samples were identified by Blast as *Alternaria alternata*.

In vitro antifungal testing of essential oils

Effect of C. nardus essential oil on the radial growth of A. brassicicola is linked to the incubation time and the oil rate in the medium

The mean values of radial mycelial growth from 2nd to

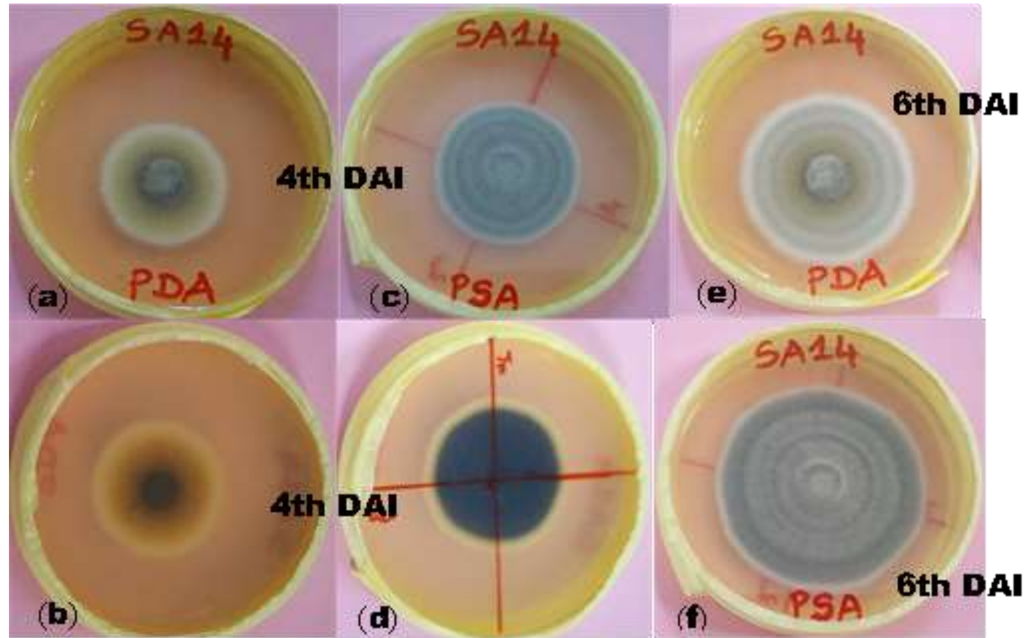

Figure 1. Macroscopic views of *A. brassicicola*, Loumbila isolate, at 4 and 6 days after incubation (**DAI**). (**a**): Colony seen on the upper side of the Petri dish containing PDA at 4th DAI. (**b**) Colony seen on the reverse side of Petri dish containing PDA at 4th DAI. (**c**): Colony seen on the top side of the Petri dish containing PSA at 4th DAI. (**d**): Colony seen on the reverse side of Petri dish containing PSA at 4th DAI. (**e**): Colony seen on the top side of Petri dish containing PDA at 6th DAI. (**f**): Colony seen on the top side of the Petri dish containing PSA at 6th DAI.

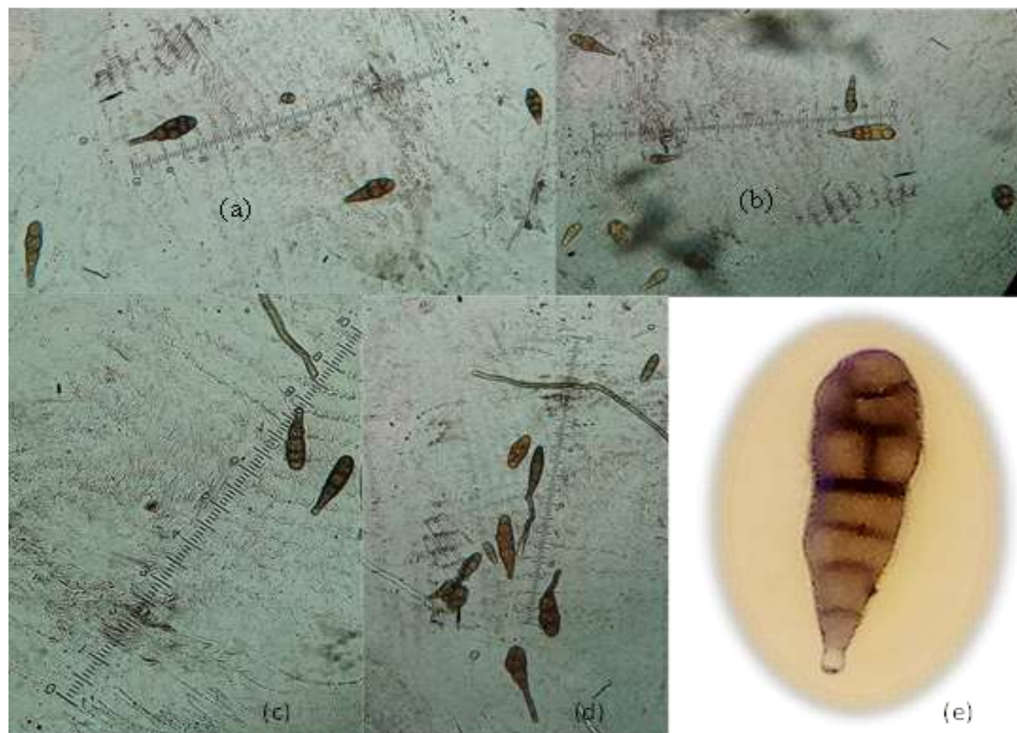

Figure 2. Conidia of *A. brassicicola* isolated from infected organs of tomato. (a) and (b): Conidia measured with a magnifying glass at 40X magnification. (c) and (d): Different forms of conidia observed under magnifying glass (40X). (e): Conidia observed with a microscope at high magnification (100X).

Table 1. Effect the treatments with the essential oil of *C. nardus* on the mycelial growth of *A. brassicicola* from day 2 to day 12 after incubation.

Treatment	Radial mycelial growth values of colonies (cm)					
	2 DAI	4 DAI	6 DAI	8 DAI	10 DAI	12 DAI
C. nardus 100	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
C. nardus 50	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
C. nardus 10	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
C. nardus 5	0.00^{A}	0.00^{A}	0.09^{A}	0.29^{ABC}	0.52^{ABCD}	0.96^{DEFG}
C. nardus 1	0.43^{ABC}	1.38^{GHI}	2.30^{K}	3.10^{MN}	3.98^{PQRS}	4.85^{V}
Mancoz.	0.00^{A}	0.06^{A}	0.18^{AB}	0.28^{ABC}	0.51^{ABCD}	0.80^{CDEF}
Azox.	0.21^{AB}	1.20^{EFGH}	2.01^{JK}	2.81^{LM}	3.66^{OPQ}	4.50^{STUV}
Control	1.74^{IJ}	3.530^{NOP}	6.08^{WX}	6.74^{YZ}	7.33^{AA}	7.98^{AB}

Table 2. Effect of treatments with *E. camaldulensis* essential oil on the mycelial growth of *A. brassicicola* over the incubation time.

Treatment	Radial mycelial growth values of colonies (cm)					
	2 DAI	4 DAI	6 DAI	8 DAI	10 DAI	12 DAI
Control	1.74^{IJ}	3.530^{NOP}	6.08^{WX}	6.74^{YZ}	7.33^{AA}	7.98^{AB}
Azox.	0.21^{AB}	1.20^{EFGH}	2.01^{JK}	2.81^{LM}	3.66^{OPQ}	4.50^{STUV}
Mancoz.	0.00^{A}	0.06^{A}	0.18^{AB}	0.28^{ABC}	0.51^{ABCD}	0.80^{CDEF}
E. camal. 100	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
E. camal. 50	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.20^{AB}
E. camal. 10	0.21^{AB}	1.03^{EFG}	1.71^{IJ}	2.44^{KL}	3.14^{MN}	3.84^{PQR}
E. camal. 5	0.44^{ABC}	1.35^{FGHI}	2.19^{K}	2.90^{M}	3.68^{OPQ}	4.31^{RSTU}
E. camal. 1	0.71^{BCDE}	1.66^{HIJ}	2.48^{KL}	3.29^{MNO}	4.09^{QRST}	4.58^{TUV}

12th DAI of the pathogen, as a function of different doses of the essential oil are presented in Table 1. The different treatments with the essential oil had a considerable and significant effect in reducing the radial mycelial growth of *A. brassicicola* over time. From the 2nd DAI to the 12th DAI no growth was observed in petri dishes treated with *C. nardus* essential oil at the 10, 50 and 100% doses. At the 5% dose, a weak growth was observed from the 6th DAI to the 12th DAI (0.09 to 0.96 cm). This growth was statistically equivalent to that recorded with the Mancozeb control and lower than that obtained with Azoxystrobin. At the 1% rate, *C. nardus* essential oil is less efficient than synthetic fungicides.

Effect of E. camaldulensis essential oil on radial growth of A. brassicicola over application time and dose

Table 2 shows the mean values of the diameter of mycelial growth of *A. brassicicola* from the 2nd to the 12th DAI, under the effect of treatments with *Eucalyptus camaldulensis* essential oil. Mean values of mycelial growth diameter ranged from 0.00 to 4.58 cm between days 2 and 12 after incubation. The untreated control recorded the highest values of mycelial growth over the incubation time. It was followed by the *E. camal.* 1% treatment, *E. camal.* 5%, and Azoxystrobin. No mycelial growth was observed over the incubation time in the case of essential oil treatments for the 50 and 100% doses (Table 2). *E. camaldulensis* essential oil diluted to 5% gave statistically equivalent values to those obtained with the fungicide Azoxystrobin.

Rate of inhibition of mycelial growth of A. brassicicola as a function of treatments 12 days after incubation

Table 3 shows the analysis of variance of the mean diameter obtained at 12th DAI under the effect of the applied treatments; and Figure 3 shows the effect of the different treatments in inhibiting mycelial growth of colonies at 12th DAI. The analysis of variance reveals highly significant differences between treatments, applied rates, but also, a significant interaction between oils and application rates ($P < 0.0001$). This means that from one dose to another the best inhibition rate may not be

Table 3. Analysis of variance of mean diameter of *A. brassicicola* as a function of treatments and doses used at the 12th IAD.

Source	DDL	Sum of squares	Average of squares	F	Pr > F
Treatments	4	183.312	45.828	374.743	< 0.0001
Doses (%)	4	122.408	30.602	250.237	< 0.0001
Treatments *Doses (%)	4	31.894	7.974	65.201	< 0.0001
Treatments *Doses (%) *Rep.	21	2.754	0.131	1.073	0.444

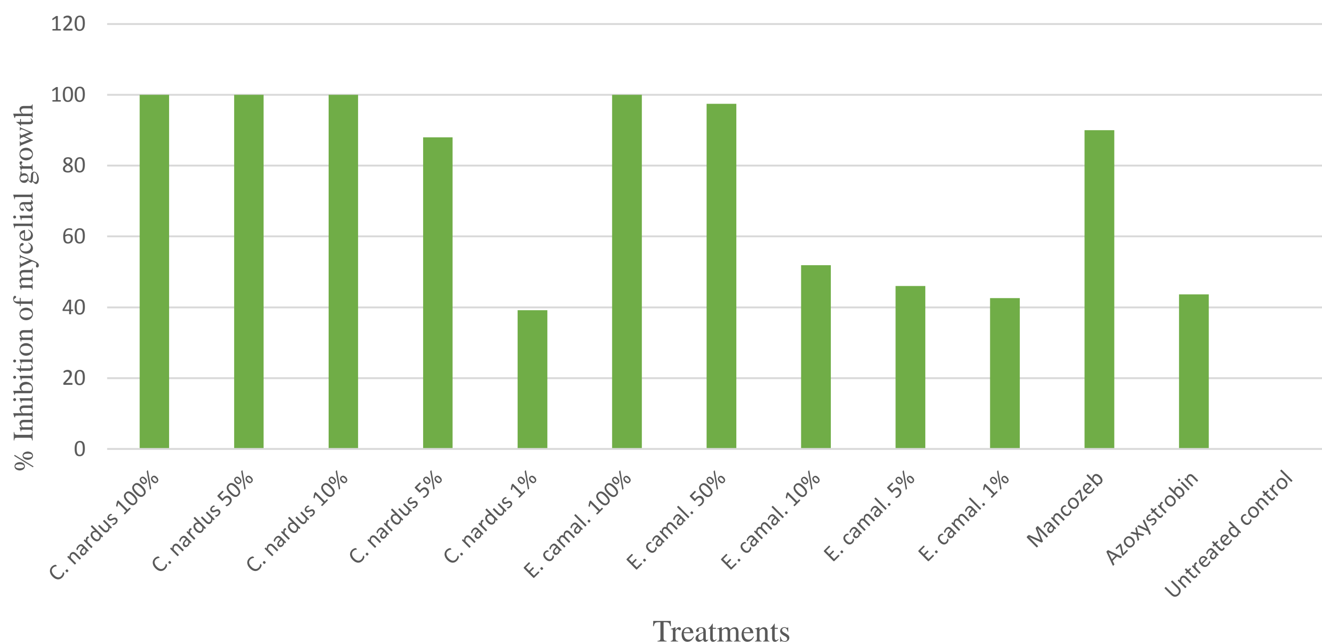

Figure 3. Effect of treatments in inhibiting mycelial growth of *Alternaria brassicicola* colonies at 12th DAI.

recorded with the same essential oil.

Treatments with *C. nardus* and *E. camaldulensis* essential oil, regardless of application rates, had an effect on reducing radial mycelial growth of the fungus in the same way as the fungicides Mancozeb and Azoxystrobin (Figure 3). However, both oils were better in inhibiting fungal growth compared to both fungicides at appropriate doses, as shown by highly significant statistical analysis ($P \leq 0.0001$) (Table 3).

DISCUSSION

The study of morphological characters of *A. brassicicola* made on both substrates (PDA and PSA) revealed that, the species had a good mycelial growth on these two substrates but with different colony staining. These observations have been previously described in several works on species of the genus Alternaria (Yu, 2015; Guo-Yin et al., 2013). Indeed, (Reddy et al., 2019) studied the morphological characters of *A. alternata* species on ten different culture media (cornmeal + Conn's Agar, Czapek's dox Agar, PDA, Malt agar, Richard's agar, V8 juice, host leaf + Agar, host stem extract, host root extract), and found that colony staining varied with the culture medium. Similarly, Singh et al. (2014) found high variability in staining on PDA medium within several isolates of *Alternaria solani*. Authors (Reis and Boiteux, 2010) have similarly observed a variation in coloration of *A. brassicicola* colonies on V8 medium as a function of the age of the culture. The genus Alternaria thus contains a high variability in colony color of the species depending on the substrate and the age of the colony. Furthermore, differences in mycelial growth as a function of medium could be explained by the difference in pH of the medium, among other factors (Khalaf, 2012).

The identity of the pathogen was confirmed on the basis of the results of rDNA sequence analysis. The results showed that the sequences of the internal transcribed spacers and the 5-8S gene of the Burkinabe isolates were identical to those of the reference isolates of the gene bank (GenBank). Detection of alternaria

species with the AAF2/AAR3 primer pair has been previously used by several authors, in the case of species such as *A. alternata*, *A. arborescens*, etc. (Konstantinova and al., 2002; Nabahat, 2014). The AAF2/AAR3 primer pair is therefore not specific but would allow detection of species with small spores related to *A. alternata*. Thus, BLAST analysis allowed to confirm the genus of the pathogen isolated from diseased organs (leaves, fruits) of tomato in Burkina Faso as *Alternaria*. The perfect match of the DNA sequences of the Burkina Faso isolates and those of GenBank could be explained by the fact that these Burkinabè isolates are of common origin. Certainly, since the fungus is a germ, the seeds (infected seeds) could be the means of transport of the species from one end of the world to the other.

Antifungal tests with plant oils from Burkina Faso (*C. nardus* and *E. camaldulensis*) showed that the oils had an identical antifungal effect on *A. brassicicola* as the chemical fungicide Mancozeb. These results underline the antifungal properties of essential oils from aromatic plants. Indeed, several authors have highlighted the antifungal activity of essential oils of *C. nardus* and *E. camaldulensis* against pathogenic germs of pets or plants in their works. Koba et al. (2004) have shown that the essential oil of *C. nardus* has antimicrobial activity against seven fungal and seven bacterial strains that cause mixed infections in dogs and cats. Aguiar et al. (2014) reported in their work that the crude component of the essential oil of *C. nardus* clearly suppressed spore production, germination and growth inhibition of *Pyricularia grisea*, *Aspergillus* spp. and *Colletotrichum musae*.

Taking into account the application dose and the time factor (duration of treatments), it appears that the essential oil of *C. nardus* at doses of 10 to 100%, and that of *E. camaldulensis* at doses of 50 and 100%, completely inhibited the development of *A. brassicicola* during the incubation time. At these doses, these two essential oils are significantly better than the chemical fungicides Mancozeb and Azoxystrobin. Similar results were found in *A. alternata* where Mancozeb, although effective on the growth of some isolates, did not completely stop their growth (Maouni et al., 2002). In addition, the essential oil of *C. nardus* was found to be the most active in inhibiting the growth of *A. brassicicola*. Indeed 10% of *C. nardus* oil was sufficient to completely inhibit mycelial growth; whereas 50% of *E. camaldulensis* essential oil was required to obtain the same effect on *A. brassicicola*. Our results are in agreement with the work of Hmiri et al. (2011), who found, using the microatmosphere method, that the essential oil of *E. camaldulensis* completely inhibited the growth of *A. alternata* and *Penicillium expansum* at the dose of 30 µl. However, at doses of 2.5, 10 and 20 µl, this oil was ineffective on these two fungi. Moreover, Kpatinvoh et al. (2017) found that the minimum inhibitory concentration (MIC) of the essential oil of *Cymbopogon citratus* against the mycoflora of alteration of cowpea, is between 0.05 and 1µl/ml on the effectiveness. Similarly, Gakuubi et al. (2017) found that the minimum inhibitory concentration and minimum fungicidal concentration of *E. camaldulensis* essential oil on Fusarium spp. were in the range of 7-8 µl/ml and 8-10 µl/ml respectively. The antifungal activity of aromatic plant oils is therefore dependent on the type of fungus and the dose of essential oil used (Touaibia, 2015; Somda et al., 2007; Yu-Chang et al., 2006). Beyond the dose effect, fungal type and duration, the difference in antifungal potency of oils may be related to their chemical compositions. Indeed, the essential oil of *E. camaldulensis* contains 62 identified compounds with 1,8-cineole (a terpene oxide) as the main component (Rabi et al., 2020; Etemadi et al., 2020). The essential oil of *C. nardus* contains mainly citronellal (30.54%), accompanied by geraniol and citronellol (Koba et al., 2009). The essential oil of *C. nardus* is predominantly composed of oxygenated monoterpenes (Koba et al., 2009), while that of *E. camaldulensis* is predominantly composed of hydrocarbon monoterpenes (Yu-Chang et al., 2006). The moderate antifungal activity of the essential oil of *E. camaldulensis* would be due to its chemical composition which differs from that of *C. nardus*. In addition, Yoshimura et al. (2011) showed that volatile monoterpenes such as 1,8-cineole had inhibitory properties, in a dose-dependent manner, on the root growth of *Brassica campestri* plants. This would explain the active action of these oils at high doses against the growth of *A. brassicicola*. Also, due to the complexity of the chemical composition of essential oils, the inhibitory activity could be related to an interaction between the different constituents of the essential oil (Mansouri et al., 2011).

Conclusion

Antifungal tests performed on the pathogen, showed that essential oils of *C. nardus* and *E. camaldulensis* when used at appropriate doses (>10%), have better antifungal properties than Mancozeb and Azoxystrobin on *A. brassicicola*. These essential oils could therefore be suitable active components in phytopesticide formulations, and constitute a credible alternative to the use of synthetic fungicides in the control of *Alternaria* species and their mycotoxins contaminating tomato.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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