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In vivo antifungal activity of neem oil and aqueous extracts against leaf spot disease caused by *Cercospora abelmoschii* on okra

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ABSTRACT: The cercospora leaf spot, caused by *Cercospora abelmoschi* Ellis and Everhart, is quite common in okra culture. Therefore, this study aimed to evaluate the efficiency of aqueous extracts of neem (*Azadirachta indica* A. Juss), citronella (*Cymbopogon nardus* (L.) Rendle), eucalyptus (*Eucalyptus grandis* L.), ecolife®, *A. indica* oil and fungicide cercobin 700 PM® in control of cercospora leaf spot on okra in greenhouse. The extracts and neem oil were tested in concentration 10%, the fungicide cercobin 700PM® in dose 2.5 $g.l^{-1}$, applied 10 days after pathogen inoculation by leaf spray and the citric biomass extract ecolife® in concentration 5.0 $ml.l^{-1}$, applied 10 days before pathogen inoculation. All treatments, except ecolife®, were effective in controlling cercospora leaf spot and may be recommended as alternatives in agroecological systems. © JASEM

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***KEYWORDS*:** *Abelmoschus esculentus*, aqueous extracts, *Cercospora abelmoschi*, fungicide cercobin and greenhouse.

INTRODUCTION

The okra (*Abelmoschus esculentus* (L.) Moench) is a vegetable appreciated by all levels of the Brazilian population. The cercospora leaf spot, caused by *Cercospora abelmoschi* Ellis and Everhart, is quite common in this culture. The presence of this fungus is especially abundant and diverse in tropical and subtropical areas (Braun et al., 2014). Therefore, on the island of São Luís, MA, Brazil, due to climatic conditions, this disease becomes one of the major for culture.

The vegetable are crops for which there is no registered agrochemicals, or there is a small number of records. As consequence, lack phytosanitary support for legalized chemical control of pests that attack (Cruz, 2013). Another problem is that even then the use of agrochemicals has increased and the their concentration in food and in our environment has caused negative effects on human health (Andersson et al., 2015).

The systematic screening of secondary metabolites of populars herbs may result in the discovery of novel and effective antimicrobial compounds (Hussain et al., 2011). Thus, this study aimed to evaluate the efficiency of aqueous extracts of neem (*Azadirachta indica* A. Juss), citronella (*Cymbopogon nardus* (L.) Rendle), eucalyptus (*Eucalyptus grandis* L.), ecolife®, neem oil and fungicide cercobin 700 PM® in control of cercospora leaf spot on okra in greenhouse.

MATERIAL AND METHODS

Leaves were collected from neem (*A. indica*), citronella (*C. nardus*) and eucalyptus (*E. grandis*) on the campus of the Universidade Estadual do Maranhão (UEMA) for analysis of macronutrients in vegetable residues. Then, they were dried, crushed, sieved, packed in plastic bags and sent to the Plant Mineral Nutrition Laboratory - UEMA for chemical analysis.

The measurements of Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca) and Magnesium (Mg) were performed by nitropercloric digestion (HNO_3 and $HClO_4$) for macronutrient (Tedesco et al., 1995). The results were obtained in $mmol_c.dm^{-3}$ for nutrients Ca, Mg, K and in $g.kg^{-1}$ for N and P.

The microbial population of each compound was analyzed according to Nakasone et al. (1999) with modifications, using aqueous extracts at 10% for total bacteria, which were added 100 ml of aqueous extracts of neem (*A. indica*), citronella (*C. nardus*) and eucalyptus (*E. grandis*) in Petri dishes containing Potato-Dextrose-Ágar (PDA) culture medium and spread with Drigalski handle. The total fungi population was obtained using 1.0 g of each residue, spread on PDA culture medium containing antibiotic.

After installation of the experiment, the plates were kept in laboratory conditions, with an approximate temperature of 25 °C and 70% relative humidity.

The evaluation was conducted by counting the colonies of total bacteria after 24h and identification of species and counting of colonies with five days of incubation for total fungi.

Visits were performed in production poles Itapera, Andiroba and Quebra Pote to collect six isolates in different varieties: Girassol (Isolate I), Valencia (Isolates II, V and VI), Santa Cruz 47 (Isolate III) and Dardo (Isolate IV), all showing characteristic symptoms of the disease.

After collecting, the material was taken to the Nucleus of Agronomic Biotechnology/NBA, Laboratory of Plant Pathology of Universidade Estadual do Maranhão for further test. The leaf tissue fragments of the middle part of the lesions were removed and submitted to the desinfestation with alcohol 50%, hypochlorite solution 1:3 and distilled water, being subsequently transferred to the culture medium PDA.

The test pathogenicity was performed in greenhouse, where okra seeds of variety "IAC-147" were sown in plastic vases with a capacity of 3 Kg, containing sterilized soil.

The experimental design was completely randomized with six treatments, corresponding to the isolates collected and seven replications, being the experimental unity, two plants per vase.

The isolates were cultured in Petri dishes containing PDA medium for one week to prepare inoculum. After this time, 20 ml of sterile distilled was added to each plate for scraping the colonies with glass slides. The inoculum was adjusted to 4 x 10^5 conidia.ml^{-1} with the aid of Neubauer chamber. The plants were inoculated 20 days after its emergence by pulverization the leaves, then placed in a moist chamber for 24h and kept in greenhouse. The test confirmation was done through observation of symptoms reproduced on okra plants inoculated with isolates.

The most virulent isolate was selected in this test and inoculated on okra plants to check the effectiveness of the treatments. To this end, we carried out a evaluation 20 days after inoculation, assigning grades 1-7, based on the diagrammatic scale Santos and Café-Filho (2005), with the following modifications: note 1 - healthy plant; note 2 - plants with up to 5% of lesions per leaf; note 3 - plant with 6 to 10% of lesions per leaf; note 4 - plants with 11 to 25% of lesions per leaf; note 5 - plants with 26 to 50% of lesions per leaf; note 6 - plants with 51 to 75% of injuries and note 7 - 76 to 90%. Data were subjected to analysis of variance and means compared by Tukey test at 5% probability.

The antifungal activity assay was installed in greenhouse of the Nucleus of Biotechnology Agronomic/NBA in the UEMA.

Initially, okra plants of the variety "IAC-147" were planted in plastic vase with 5 kg capacity, containing autoclaved soil.

The inocullum was obtained from the collection of conidia of foliar lesions, with sterile distilled water and with a brush, and subsequently filtered through double cheesecloth and the concentration adjusted to 4 x 10^5 conidia.ml^{-1}. Inoculation of selected pathogen in pathogenicity test was performed 20 days after germination, through foliar spray, keeping the plants moist chamber for 24h.

The treatments consisted of dosages of aqueous extracts of neem, citronella and eucalyptus, neem oil, in the concentration 10%, of the fungicide cercobin 700 PM® in the dose 2.5 g.l^{-1}, applied 10 days after inoculation of the pathogen, by foliar spray and ecolife® extract in the concentration 5.0 ml.l^{-1}, applied to 10 days before the pathogen inoculation. The control was inoculated with the pathogen and sprayed with distilled water only.

The evaluation of severity was carried out 20 days after treatment application, based on the diagrammatic scale Santos and Café-Filho (2005), described above.

The experimental design was completely randomized with seven treatments and five replicates and each experimental unit consisted of a vase containing two plants. The data were subjected to analysis of variance by Assistat program (Silva and Azevedo, 2002) and means compared by Tukey test at 5% probability.

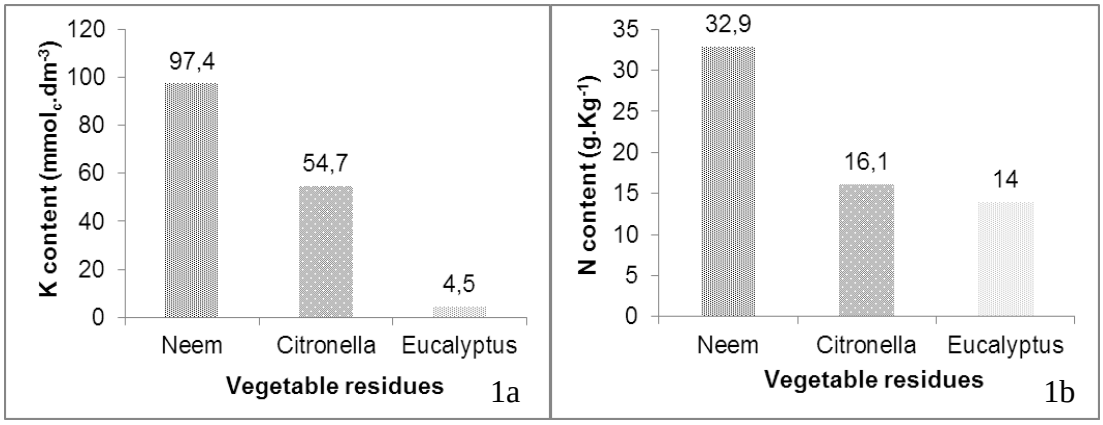
To calculate the percentage of control efficiency of the treatments, we used the Abbott's formula (Abbott, 1925):

$$E = C - T \times 100/C$$, were:

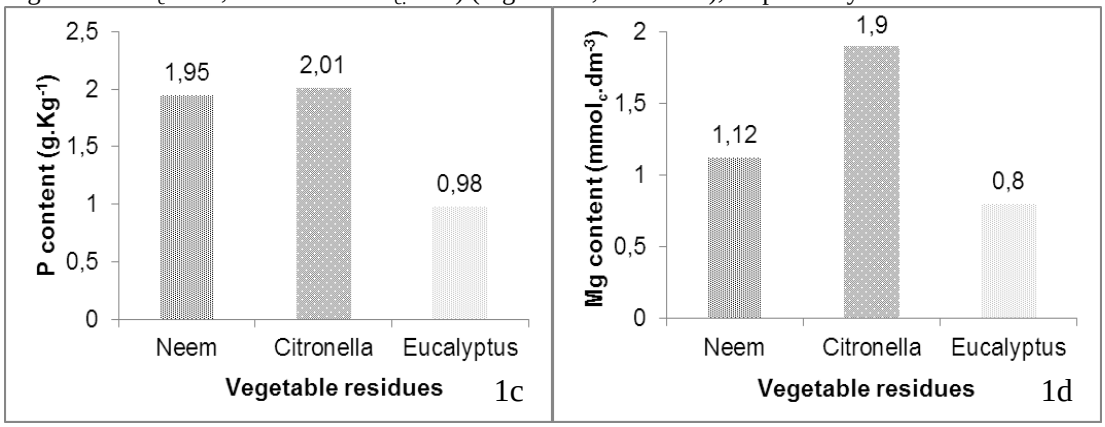
E: Percentage of control efficiency
C: disease severity in control
T: disease severity in treatment

RESULTS AND DISCUSSION

The nutrients found in greater quantity in the residues were N (Figure 1a) and K (Figure 1b). Highest levels of these nutrients were found in neem residue (N=32.9 g.Kg^{-1}, K=97.4 mmol$_c$.dm^{-3}), followed by citronella (N=16.1 g.kg^{-1}; K=54.7 mmol$_c$.dm^{-3}) and eucalyptus (N=14 g.kg^{-1}; K=4.5 mmol$_c$.dm^{-3}) (Figures 1a and 1b).



The macronutrients P, Mg and Ca were observed in highest amount in citronella residue (P=2.01 g.Kg^{-1}; Mg=1.9 mmol$_c$.dm^{-3}, Ca=2.06 mmol$_c$.dm^{-3}) (Figures 1c, 1d and 1e), respectively.



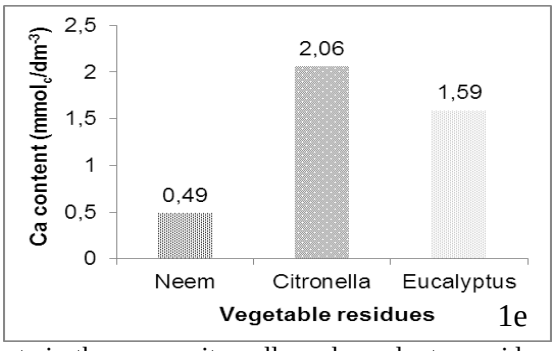


Fig 1. Content of macronutrients in the neem, citronella and eucalyptus residues. 1a. potassium; 1b. nitrogen; 1c. phosphorus; 1d. magnesium and 1e. calcium.

The nutrition of okra is characterized by an extraction of macro and micronutrients slow until 20 days, thereafter been coming to increase (Cavalcante et al., 2010). In our work, the aqueous extracts of neem, citronella, eucalyptus and neem oil application by foliar spraying in plant okra to the 30 days of age, may have contributed to a better development of culture and also for increasing their resistance. This increase may occur by changes in the anatomy, such the presence of thick cuticle and plasma membrane, lignin and other substances available in different parts of the plants or by a reaction of external or internal stimulus, generally characterized by hypersensitivity reaction (HR), or survival of the plant to pathogen attack after being properly identified or recognized infection (Fancelli, 2008).

In the analysis of the microbial population, the neem residue exhibited a larger number of bacterial colonies, followed by citronella and eucalyptus (Table 1).

Table 1. Total bacteria detected in aqueous extracts of neem, citronella and eucalyptus.

Extracts	N° of colonies* (100 µl)	Frequency
Neem	240	44.44
Citronella	170	31,48
Eucalyptus	130	24,07
Total	540	100

*Average of five plates/residues.

The presence of bacteria in the extracts may trigger a series of reactions that can directly influence the presence of pathogens in the host and activate the plant defense mechanisms (Oliveira et al., 2003). Studies have shown that use of this antagonistic contribute greatly to the control of plant pathogens, including the fungi species control that cause cercospora leaf spot, such as *C. sojina* (Tonelli and Fabra, 2014) and *C. carthami* (Govindappa et al., 2014).

In evaluation total fungi, the species that occurred more frequently were *Mucor* sp., *Aspergillus niger*, *Rhizopus stolonifer* and *A. ochraceus* (Figure 2), whereas the *Mucor* sp. presented the greatest number of colonies (Figure 2). In the neem residue was detected the larger number of fungal colonies, followed citronella and eucalyptus, and only in this latter it was verified the presence of *A. ochraceus* (Figure 2).

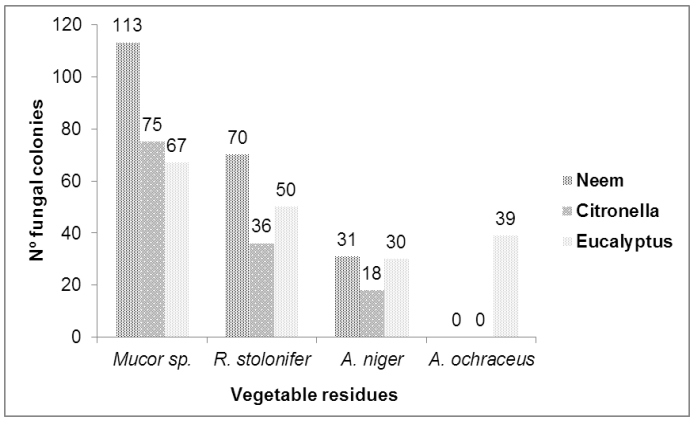


Fig 2. Number fungal colonies detected in neem, citronella e eucalyptus residues.

All these genera have been reported for other microorganisms controlling on biocontrol tests (Bettiol et al., 2012; kusumaningtyas et al., 2006; Ziedan et al., 2013). Moreover, there are many products already market based on fungi for the biological control of pathogens in plants, whose effectiveness is proven (Bettiol et al., 2012).

In the pathogenicity test, we can observe the symptoms of the disease on inoculated okra plants, confirming the test. Among the six isolates tested in variety of okra "IAC-147," the isolate IV appeared more virulent (Figure 3). With this, the effect of the treatments was tested against this isolated.

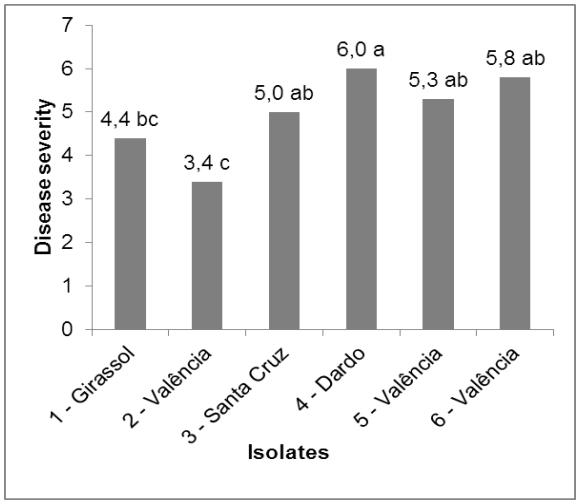


Fig 3. Pathogenicity test of *C. abelmoschi* isolates on okra.

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Statistically there was no difference between the neem treatments (3.0b), eucalyptus (3.4b), citronella (3.2b), neem oil (3.4b) and fungicide (3.2b), although neem extract has providing a greater percentage of control efficiency than the others (42.31%). It was observed that neem oil and eucalyptus extract had the same control efficiency percentages (34.62%) and citronella and fungicide treatments, which showed equal values to 38.46%. Unlike, when using a citric biomass extract ecolife® there was little reduction of the disease and the results did not differ statistically from the witness (5.2a) with a percentage of 15.38% control efficiency (4.4ab) (Table 2).

Table 2. Effect of treatments on cercospora leaf spot severity on okra

Treatments	Disease severity	E[3]
Neem 10%	3.0b[1]	42,31
Citronella 10%	3.2b[1]	38,46
Cercobin 700 PM® 2.5 $g.l^{-1}$	3.2b[1]	38,46
Eucalyptus 10%	3.4b[1]	34,62
Neem oil 10%	3.4b[1]	34,62
Ecolife® 5.0 $ml.l^{-1}$	4.4ab[1,2]	15,38
Water	5.2a[2]	-
Mean	3.70	
F	5.70*	
CV%	20,51	

[1,2]means followed by the same letter (s) are not significantly different at 0.05 level of probability according to Tukey's Test.
[3]Data obtained by applying the Abbott's formula: E = C - T × 100/C.
*Significant at 0.05 level of probability.

The use of natural products as an alternative for disease control is an attractive tool and desired by the population. There is at least some evidence that natural products such as essential oil and plants extracts may cause less deleterious effects than corresponding synthetic drugs (Tabassum and Vidyasagar, 2013).

Indeed, in this work, the presence of nutrients and microorganisms in the residues become its most effective for the control of cercospora leaf spot. The results of the analysis indicate the potential of organic residue on fertility of the plants and in biological control. Aiming to minimize the negative effects of pesticides has been development a alternative form of control of plant disease, which includes the biological control, the induction of resistance and the use of natural products with induction of resistance and/or with direct antimicrobial activities (Stangarlin et al., 2011).

We recommend the use of natural products tested, for which satisfactory results were obtained. This study, like several others, has shown that the use of natural products is an alternative that can replace the use of pesticides, as it allows an effective control of diseases, without harming the ecosystem.

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REFERENCES

Abbott, W S (1925). A method of computing the effectiveness of an inseticide. J Econ Entom 18: 265 - 267.

Andersson, H; Tago, D; Treich, N (2015). "Pesticides and health: A review of evidence on health effects, valuation of risks, and benefit-cost analysis". Adv Health Econ Health Serv Res 5: 1 - 61.

Bettiol, W; Morandi, M A B; Pinto, Z V; Paula Júnior, T J de; Corrêa, E B; Moura, A B; Lucon, C M M; Costa, J C B; Bezerra, J L (2012). Produtos comerciais à base de agentes de biocontrole de doenças de plantas. Embrapa Meio Amb 88: 1 - 155.

Braun, U; Crous, P W; Nakashima, C (2014). Cercosporoid fungi (*Mycosphaerellaceae*) 2. species on monocots (Acoraceae to Xyridaceae, excluding Poaceae). IMA Fungus 5: 203 - 390.

Cavalcante, L F; Diniz, A A; Santos, L C F dos; Rebequi, A M; Nunes, J C; Brehm, M A S (2010). Teores foliares de macronutrientes em quiabeiro cultivado sob diferentes fontes e níveis de matéria orgânica. Semina: Ciênc Agrár 31: 19 - 28.

Cruz, D (2013). As hortaliças e o registro de agrotóxicos. Hortic Bras 31: 1.

Fancelli, A L (2008). Influência da nutrição na ocorrência de doenças de plantas. Inf Agron 122: 23 - 24.

Govindappa, M; Rai, V R; Lokesh, S (2014). Induction of resistance against cercospora leaf

spot in safflower by seed treatment with plant growth-promoting rhizobacteria. Arch Phytopathology Plant Protect 47: 2479 - 2492.

Hussain, I A; Anwar, F; Nigam, P S; Sarker, S D; Moore, J E; Rao, J R; Mazumdar, A (2011). Antibacterial activity of some Lamiaceae essential oils using resazurin as an indicator of cell growth. Food Sci Technol 44: 1199 - 1206.

Kusumaningtyas, E; Widiastuti, R; Maryam, R (2006). Reduction of aflatoxin B1 in chicken feed by using *Saccharomyces cerevisiae*, *Rhizopus oligosporus* and their combination. Mycopathol 162: 307 – 311.

Nakasone, A K; Bettiol, W; Souza, R M (1999). Efeito de extratos aquosos de matéria orgânica sobre fitopatógenos. Summa Phytopathol 25: 330 - 335.

Oliveira, A L M; Urquiaga, S; Baldani, J I (2003). Processos e mecanismos envolvidos na influência de microrganismos sobre o crescimento vegetal. Embrapa Agrobiol 161: 1 - 40.

Santos, G R; Café-Filho, A C (2005). Reação de genótipos de melancia ao crestamento gomoso do caule mela. Hortic Bras 23: 945 - 950.

Silva, F A S; Azevedo, C A V (2002). Versão do programa computacional Assistat para o sistema operacional Windows. Rev Bras Prod Agroind 4: 71-78, 2002.

Stangarlin, J R; Kuhn, O J; Assi, L; Schwan-Estrada, K R F (2011). Control of plant diseases using extracts from medicinal plants and fungi. In: Mendéz-Vilas, A (ed) Science against microbial pathogens: communicating current research and technological advances, Formatex, Extremadura, p1033-1042.

Tabassum, N; Vidyasagar, G M (2013). Antifungal investigations on plant essential oils. A review. Int J Pharm Sci 5: 19 - 28.

Tedesco, M J; Gianello, C; Bissani, C A; Bohnen, H; Volkweiss, S J (1995). Análises de solo, plantas e outros materiais. Universidade Federal do Rio Grande do Sul, Porto Alegre, RS p89.

Tonelli, M L; Fabra, A (2014). The biocontrol agent *Bacillus* sp. CHEP5 primes the defense response against *Cercospora sojina*. World J Microbiol Biotechnol 30: 2503 - 2509.

Ziedan, E H; Farrag (Eman), S H; Sahab, A F (2013). First record and preliminary evaluation of *Mucor hiemalis* as biocontrol agent on inflorescence brown rot incidence of date palm. Arch Phytopathology Plant Protect 46: 617 - 626.