P-ISSN: 3009 – 7487
E-ISSN: 3009 – 788

JOURNAL OF PLANT AND FOOD SCIENCES December, 2023

Suppression and Molecular identification of severe *Cercospora beticola* causing Cercospora leaf spot disease on sugar beet

Mohamed D. Sehsah[1], Samar S.A. Elsayed[1], Walaa R. Abdelghany[1], Heba I. Saad El-Din, [1], Heba Afifi [2] and Reda I. Omara[1]

[1] Plant Pathology Research Institute, Agricultural Research Center, Giza 12619, Egypt
[2] Gene Bank, Agriculture Research Center, Giza, Egypt.
***Correspondence:** **Omara, R. I.** (redaomara43@arc.sci.eg; https://orcid.org/0000-0002-9776-0238)
Received: 1 November 2023; in revised form: 21 November 2023 / Accepted: 1 December 2023 / Published: 31 December 2023
DOI 10.21608/JPFS.2023.259788.1006

ABSTRACT

Cercospora leaf spot disease, triggered by *Cercospora beticola*, poses a substantial threat to sugar beet varieties. Evaluation of nineteen sugar beet varieties indicated that Perfekta, Smart meyra KWS, Aseel, and Vulkhan exhibited the lowest disease severity and incidence. At the same time, Polylias, BTS 3880, Prilive, Faralda Kws, Zoom, and Smart Seza KWS showed higher susceptibility. The causal organism was isolated from diseased sugar beet plants in Kafrelshiekh, Behira, and Dakahlia governorates during the 2020 growing season. Twenty-seven fungal isolates were obtained and evaluated for aggressiveness on susceptible sugar beet variety (Polylias cv). Phylogenetic analysis using ITS sequences revealed genetic variations among the four most aggressive isolates, which were subsequently registered in NCBI. Furthermore, the efficacy of essential oils Citronella and Rose Geranium (*Pelargonium graveolens*) in controlling *C. beticola* was examined. *Pelargonium graveolens* at 10 ml/L demonstrated the highest effectiveness in reducing fungal growth, as well as in increasing total soluble solids content and sucrose. Additionally, this essential oil enhanced the activity of polyphenol oxidase and peroxidase enzymes. Scanning electron microscopy (SEM) examination confirmed that Citronella and *Pelargonium graveolens*, along with the fungicide montoro 30%, induced plasmolysis, decomposition, and damage to conidiophores and conidiospores of *C. beticola*, contrasted to the control treatment. This integrated approach, combining molecular characterization, disease evaluation, and essential oil application, provides valuable insights for the management of Cercospora leaf spot on sugar beet, offering sustainable and environmentally friendly alternatives to traditional fungicides.

Keywords: *Cercospora beticola*, *Pelargonium graveolens*, *Cymbopogon citratus*, Citronella, Rose geranium, ITS sequence.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) holds a crucial position in global agriculture as a valuable crop with significant economic and industrial importance. However, the productivity and profitability of sugar beet cultivation can be severely impacted by plant pathogens, which have the potential to substantially reduce crop yields. Among the various diseases affecting sugar beet, one of the most economically destructive foliar diseases is Cercospora leaf spot (CLS), triggered by *Cercospora beticola* (Sacc), leading to considerable economic losses (Muellender *et al*., 2021).

The epidemiological behavior of *C. beticola* is dictated by a variety of circumstances, including the predisposition of sugar beet plants to infection. Variations in predisposition can significantly affect the severity and spread of CLS within sugar beet fields. Furthermore, the dynamics of *C. beticola* populations are also subject to environmental factors, such as climate change. Climate change, as a meteorological variable, has the potential to disrupt the delicate balance between sugar beet plants and *C. beticola*. Alterations in temperature, humidity, and precipitation patterns can impact the stages and rate of development of the pathogen. Additionally, climate change may influence host plant resistance mechanisms and alter the physiological interactions between sugar beet plants and *C. beticola* (Khalil *et al*., 2007). By elucidating the mechanisms underlying disease development and spread, researchers can work towards implementing sustainable and resilient practices to reduce the impact of plant pathogens on sugar beet yields. Such efforts are crucial for ensuring the continued productivity and profitability of sugar beet farming in the face of evolving environmental challenges.

Genetic diversity in *C. beticola* isolates gathered from diverse regions of Iran was highlighted by Mahmoudi *et al*. (2018), emphasizing the need for effective detection methods. Frequent fungicide usage can potentially lead to fungal disease resistance, posing risks to the environment and normal flora. Over recent years, there has been a growing inclination among the general public toward the utilization of medicinal plants over synthetic drugs (Balahbib *et al*., 2020). Essential oils from plants, such as *Pelargonium graveolens*, have the potential to control plant diseases. The chemical profile of *Pelargonium graveolens* essential oil, including geraniol, citronella, linalool, and γ-eudesmol, indicates its potential use in controlling *Botrytis cinerea* in rose flowers (María *et al*., 2022).

Consequently, essential oils such as lemongrass, have shown promising antifungal properties, severely damaging conidia of *C. musae* and *C. gloeosporioides* and preventing germination (Rozwalka *et al*., 2010). Additionally, essential oils like Citral and Methyl anthranate have demonstrated the potential to increase sugar beet yield and total soluble solids content (Fatouh *et al*., 2011). Essential oil ingredients might be employed as environmentally friendly natural substances to prevent CLS infections in sugar beet plants in the field. Studies of employing essential oils on the physiological processes inside plants have emphasized the influence of particular enzymes, such as polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase, in response to disease control substances (Desoky *et al*., 2020).

Scanning electron microscopy (SEM) has been instrumental in studying fungal growth and its interactions with plant fragments (Jackowiak *et al*., 2005). Citronella essential oil has shown effectiveness in managing brown eye spots in coffee plants, activating the plant's defense system and increasing peroxidase (POX) and chitinase (CHI) activities, along with lignin accumulation (Pereira *et al*., 2012; Omara *et al*., 2020; Elsharkawy e*t al*., 2022). In a separate study, citronella oil demonstrated destructive effects on *A. niger* hyphae, penetrating the cell membrane and causing significant damage to organelles due to large cytoplasm loss, indicating its potential as an environmentally friendly fungicide (Wen-Ru *et al*., 2013). Chen *et al*. (2014) reported citronella oil's inhibitory effects on *A. alternata*, suggesting its potential for controlling black rot in cherry tomatoes revealing considerably abnormal mycelial morphology. Nehal *et al*. (2015) found that foliar application of garlic, lemongrass, and basil extracts reduced *T. absoluta* populations and improved tomato fruit yield.

Essential oils, including lemongrass oil from *Cymbopogon citratus*, have been studied for their antifungal properties against various plant pathogens (Rabari *et al*., 2018; Sharma *et al*., 2018). Commercial cultivation of essential oil-bearing Cymbopogon species serves multiple purposes in tropical regions (Camacho *et al*., 2015). Ghazi *et al*. (2018) demonstrated that essential oils, including citronella, reduced charcoal rot severity and enhanced plant growth parameters. Mark Angelo *et al*. (2019) highlighted the antifungal properties of Cymbopogon essential oils against various pathogens, affecting conidia germination in *C. gloeosporioides*. Their research revealed that these oils can effectively inhibit the growth of several pathogens

including *Fusarium oxysporum* (causing banana wilt), *Colletotrichum gloeosporioides* (responsible for mango fruit anthracnose), *C. falcatum* (associated with sugarcane red rot), and *Neopestalotiopsis* spp. (linked to mango leaf spot). This study sheds light on the wide-ranging potential of citronella essential oil in controlling plant diseases. Furthermore, their findings suggest that they negatively impact the germination of conidia and elongation of germ tubes in *C. gloeosporioides*, underscoring its significant influence on the reproductive biology of this pathogen.

In a similar vein, Sehsah *et al*. (2022) demonstrated that treatments with natural bioagents significantly improved the activity of phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase in sugar beet plants. These environmentally friendly treatments showed efficacy against *C. beticola in vitro* and *in vivo*, reducing the severity of CLS and enhancing sugar beet qualities. Therefore, the objective of this study is to molecularly identify *C. beticola* isolates and assess the efficacy of some substances such as *Pelargonium graveolens* and citronella against CLS on sugar beet. The effects are examined using scanning electron microscopy (SEM) and by estimating oxidative enzymes, including peroxidase and polyphenol oxidases.

MATERIALS AND METHODS

Evaluation of sugar beet cultivars against CLS disease:

The assessment of nineteen sugar beet cultivars against CLS disease was conducted at Sakha Agricultural Research Station over two seasons (2019/2020 and 2020/2021). The experiment was employed by randomized complect block design with three replicates, and each replicate was sown with 19 sugar beet cultivars. The experimental unit, representing each cultivar, was planted in two rows, each 5m in length and spaced 60 cm apart. Within each row, 25 hills were planted at intervals of 20 cm. After 120 days from sowing, disease incidence (D.I. = Mean no. of spots/leaf) and disease severity % (D.S.= % of infected surface area.) were determined following the methodology outlined by Shane and Teng (1992).

Collection, isolation, and identification of *C. beticola:*

Sugar beet leaves displaying symptoms of leaf spot disease triggered by *C. beticola* were gathered from the North Delta restriction, encompassing Kafrelshiekh, Behira, and Dakahlia governorates. The leaves were thoroughly washed with tap water, finely chopped, and subjected to surface sterilization for three minutes in a 0.5% sodium hypochlorite solution, following the protocol detailed by Alaniz *et al*. (2011). The collected infected sugar beet leaves were introduced to Petri plates containing Sugar Beet Leaf Extracts Dextrose Agar media (SBLEDA) media, incubated at 27 ± 2 °C for 5 days, and subjected to regular examinations to monitor fungal growth. The developing fungi underwent microscopic scrutiny and purification by the hyphal tip technique outlined by Barnett and Hunter (1972). The purified isolates were stored on PDA slants at 4 °C for subsequent analysis. Isolates were identified using morphological and microscopic features, following the criteria set by Barnett and Hunter (1972).

Screening aggressiveness of collected *C. beticola* isolates:

In a greenhouse setting, a pathogenicity test was conducted in 40-cm-diameter pots filled with sandy-loam soil (1:2 w/w ratio). Twenty-seven isolates of *C. beticola* were assessed for their impact on the susceptible Polylias sugar beet cultivar, concerning CLS infection. The isolates were grown in liquid CZ-a pek medium and kept at 27 ± 3 °C for 15 days to produce the required inoculum. Ninety-day-old plants were subjected to spraying with 5×10^4 spores/ml of each strain using an atomizer (Crane and Calpouzos, 1984), with four repetitions, each consisting of four plants. Before inoculation, plants were sprayed with water to form a thin layer on the leaf surface. To promote infection, 2 grams of sucrose and 0.1 ml of Tween 80 per liter were included in the spore suspension. The inoculated plants were then housed in a humid polyethylene chamber for seven days. Conversely, disease incidence (D.I.) and disease severity

% (D.S.) were calculated as described by Shane and Teng (1992), 100 days after planting. The highly aggressive isolates were specifically chosen from the original group of 27 tested strains and stored for future research at the NGB.

Molecular characterization:

Extraction of genomic DNA from *Cercospora beticola* selected isolates:

The genomic DNA of the phenotypically identified *Cercospora beticola* isolates was extracted from growing mycelium. The procedures were carried out according to manufacture instructions of the QIAGEN DNeasy Plant Mini Kit adapted from (www.qiagen.com/HB-1166). Steps of lysis, extraction buffers and purifications in the supplied column through many centrifugation intervals were added directly after the previous grinding step. At the end of the procedure, around 100 µl of extracted DNA was eluted.

PCR amplification of fungal ITS1 and/ ITS4 rDNA gene regions:

Four isolates were taken up for PCR amplification to be genetically identified using ITS1 (TCTGTAGGTGAACCTGCGG) & ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al*., 1990). The genomic DNA was amplified using PCR technique. The PCR protocol began with an initial denaturation step at 94°C for 8 minutes, followed by 32 cycles of denaturation at 94°C for 1 minute, annealing at 51°C for 30 seconds, and extension at 70°C for 2 minutes. Finally, a final extension step was conducted at 72°C for 7 minutes using a BIOER/Life ECO 96 advanced gradient Thermocycler. Following amplification, 20 µl of the PCR product was mixed with a loading buffer (8 µl) comprising 0.25% bromophenol blue and 40% w/v sucrose in water. This mixture was then loaded onto a 2% agarose gel containing 0.1% ethidium bromide for visualization using horizontal electrophoresis.

Nucleotide sequencing analysis

The ITS PCR products were sequenced using the Sanger chain-termination method, with sequencing performed at least twice for one direction, utilizing primers. The sequencing data were compiled and compared to databases using the BLAST server on the NCBI Web site [http://www.ncbi.nlm.nih.gov/BLAST], as well as the Mega 11 program and TreeView programs for further analysis and comparison.

In vitro screening of essential oils against *C. beticola*:

The efficacy of two essential oils, Citronella, and Rose Geranium, against *C. beticola* was evaluated through a completely randomized approach. Treatment effectiveness was assessed by calculating the percent decrease in linear growth parallel to the untreated control. Two concentrations, 5 and 10 ml/L, of the tested materials were prepared by adding the required volume of stock solution to 60 ml volumes of sterilized PDA media that had been chilled to approximately 45 °C. The necessary quantities of the two essential oils (Citronella and Rose Geranium) and Montoro fungicide were added to PDA petri plates. Each treatment concentration had four Petri plates (10 ml) as replicates. A control treatment without any additives was also included. In each Petri plate, a 15-d *C. beticola* disc (5 mm in diameter) was introduced for inoculation. The Petri dishes were sealed with parafilm to avoid the evaporation of volatile substances. Subsequently, the dishes were then left to incubate at 22–25 °C until the control treatment exhibited full growth at which juncture the linear growth was measured according to Ibrahim *et al.* (1987). Essential oils Citronella (*Cymbopogon citratus*) and Rose geranium (*Pelargonium graveolens*) were obtained from Sakha Horticultural Research Station – Kafrelshiekh- Egypt. The reduction of linear growth % was estimated according as follows:

$$R = \frac{C - T}{C} \times 100$$

R = % of reduction of fungi development.
C = linear development of untreated plates.
T = linear development of treated plates.

Assessment of essential oils against *Cercospora beticola* in the field:

A randomized complete block design involving three replicates was implemented over two growing seasons (2020/2021) and (2021/2022) at the Plant Pathology Research Institute's, Sakha Agricultural Research Station, Kafrelshiekh, Egypt. The design included six rows per replication, each 5 meters long and 60 cm wide, with 25 hills in each row spaced 20 cm apart. The experimental unit consisted of two rows (6 m^2), utilizing the susceptible sugar beet cultivar Polylias. Standard cultural practices were followed at optimal times. Upon the discovery of illness symptoms, spraying commenced (after 90 days of cultivation). Plants underwent three rounds of spraying at ten-day intervals with two essential oils, Citronella (*Cymbopogon citratus*) at 10 ml/L and Rose geranium (*Pelargonium graveolens*) at 10 ml/L, along with the recommended fungicide Montoro at one ml/L. This fungicide consists of Propiconazole 15% + Difenoconazole 15%, marketed as Montoro 30% EC, and was used at its recommended field rate of 1 ml/L. A control plot was left untreated. Disease severity was assessed three times, ten days following each spray, using the method outlined by Shane and Teng (1992). The assessment of Total Soluble Solids content (TSS%) and sucrose percentage in freshly harvested sugar beet roots was conducted using a hand refractometer and saccharometer, respectively. The procedures for these measurements followed the protocols outlined by the Association of Official Analytical Chemistry (A.O.A.C.) (1990) and McGinnis (1982).

Determination of polyphenol oxidase and peroxidase enzyme activity:

Enzyme extraction and assay procedures were performed approximately 24 hours post-spraying, involving the collection of leaf samples from separate treatment, encompassing both healthy and infected specimens. The harvested leaf tissues were subsequently ground in a porcelain mortar using 0.1 M sodium phosphate buffer at pH 7.1 (2 g of leaf tissues per mL). The extracted enzymes were then filtered through four layers of cheesecloth, followed by centrifugation at 6°C for 20 minutes at 3000 rpm. The resulting clear supernatants were gathered as crude enzyme extracts. To evaluate peroxidase activity, we employed the method described by Allam *et al*. (1972), measuring the oxidation of pyrogallol to pyrogalline in the presence of hydrogen peroxide. Changes in absorbance at 425 nm were recorded at 1-minute intervals for a duration of 4 minutes. Furthermore, polyphenol oxidase activity was determined by measuring changes in absorbance at 495 nm spectrophotometrically, recorded every 1 minute over a 4-minute period. The Beckman Spectrophotometer Du_7400 was utilized for all measurements.

Scanning electron microscopy (SEM) investigation of the interaction between essential oils, Montoro fungicide and *C. beticola* on sugar beet leaves:

The assessment of the impact of treatments on the size and number of spots on infected sugar beet leaves, as well as the production of C. beticola conidiospores and spores, was conducted following the methodology described by Manzali *et al.* (1993). In the Electron Microscope Unit at Nanotechnology Institute, Kafrelsheikh University, interaction sites (lesions) were identified. Disc blocks of 1 cm^2 were excised from these interaction regions for subsequent scanning electron microscopy (SEM) analysis using a Jeol Scanning Electron Microscope model JSM-5500lv. The interaction region underwent fixation with osmium oxide, dehydration through a series of ethyl alcohol dilutions and acetone, and subsequent drying with a critical point drier (EMS 850). Following these steps, the samples were coated with gold using a sputter coater (EMS 550) and subjected to examination using a SEM (Jeol 100cx-11 ASID-4D).

Statistical analysis

The statistical analysis, as outlined by Gomez and Gomez (1984), included the application of analysis of variance (ANOVA), and subsequent testing of means using the least significant difference test (LSD).

RESULTS AND DISCUSSION

Evaluation of sugar beet cultivars against CLS disease:

Nineteen sugar beet cultivars were assessed for their susceptibility to the CLS disease caused by *C. beticola*. The evaluation, as presented in Table (1) revealed varying levels of resistance among the cultivars under field conditions. There was a high difference in disease severity and disease incidence between the tested sugar beet cultivars during the two tested seasons. Therefore, the cultivars Polylias, Faralda Kws, BTS 3880**,** Smart Seza KWS**,** Zoom, and Prilive had the highest disease incidence and disease severity%; they ranged from 11 to 16.333 and from 9.00 to 13.333%, respectively, of 2020 season. While the rest of cultivars had disease incidences of less than 10 and ranged from 2.333 to 9.333, the disease severity was also less than 10% and ranged from 0.666 to 9.000% during the growing season 2020. The results obtained from the 2021 growing season followed the same trend as those obtained during the 2020 growing season. The severity and incidence of the disease displayed noteworthy variations among the evaluated sugar beet cultivars across the two testing growing seasons. These findings were consistent with Khalil *et al*. (2007) observations, indicating that predisposition variation influences the epidemiological behavior of *C. beticola*. Additionally, the meteorological factor of climate change was acknowledged for its potential alterations in the pathogen stages, rate of development, host resistance, and changes in host-pathogen interactions.

Table 1: Evaluation of nineteen cultivars against CLS during the 2020 and 2021 growing seasons.

Entry	Sugar Beet cultivars	Season 2020		Season 2021	
		Disease incidence (D.I.)	Disease severity % (D. S.%)	Disease incidence (D.I.)	Disease severity% (D. S.%)
1	Volna	5.333	3.333	5.666	4.333
2	BTS8935	6.333	5.666	7.333	6.666
3	Perfekta	2.333	0.666	2.333	1.666
4	BTS 3880	13,666	10.666	13.333	11.666
5	Polylias	16,333	13.333	15.666	13.000
6	Symbol	7.333	5.666	8.333	6.666
7	Prilive	11.000	9.000	11.666	9.666
8	Afendra Kws	7.666	6.333	8.666	7.333
9	Faralda Kws	16.000	13.333	15.666	13.333
10	Zoom	12,333	10.666	13.333	10.666
11	Collin	7.666	5.666	8.333	6.333
12	BTS 3740	8.000	5.000	8.333	6.000
13	Burya	9.333	6.333	8.666	6.666
14	Smart meyra KWS	4.666	2.333	5.333	3.333
15	Stikhiya	5.333	3.000	5.666	3.666
16	Aseel	4.666	2.000	5.666	3.000
17	Dell 1135	6.333	4.333	7.333	5.333
18	Smart seza KWS	13.333	11.666	15 666	12.666
19	Vulkhan	4.666	3.000	5.333	3.666
L.S.D$_{0.05}$		**0.602**	**3.144**	**1.090**	**2.535**

The trials were conducted to isolate, identify, and evaluate sugar beet cultivars against *Cercospora beticola*. Diseased sugar beet plants with leaf spots were collected from the North Delta regions, including the Kafrelshiekh, Behira, and Dakahlia governorates, resulting in the isolation of twenty-seven isolates. They were identified as *C. beticola* based on their morphological and microscopic characteristics. An experiment was then carried out to assess the aggressiveness of these isolates on a susceptible sugar beet variety (Polylias cv.) in

screening for CLS disease. The susceptible sugar beet variety was artificially infected with $5x10^4$ spores/ml of *C. beticola* 90 d after cultivation. After two weeks of infection, the incidence and severity of the disease were evaluated as previously described. The results obtained in Table (2) indicate that isolates 2, 6, 17, 18, 21, 22, 24, and 27 had the highest disease severity % on the tested susceptible beet varieties and ranged from 12.666 to 14.666 %, while isolates 9, 14, 19, and 25 had the lowest disease severity % and ranged from 7.333 to 7.666 %. The data obtained in Table (2) show the same trend in the case of disease incidence. The results highlighted substantial variation among the twenty-seven fungal isolates in terms of their impact on Cercospora leaf spot disease symptoms. As outlined by Mahmoudi *et al*. (2018), an investigation into the genotypic and pathogenic variation of 24 *C. beticola* isolates from diverse regions in Iran revealed a notable level of genetic diversity. This observation suggests variations in the detection methods for *C. beticola*. In contrast, farmers commonly turn to chemical pesticides as their initial defense strategy against plant diseases. Unfortunately, these pesticides contain potent chemicals that indiscriminately eliminate both harmful and beneficial bacteria. Moreover, they lead to the eradication of numerous environmentally beneficial microbes, disrupting the ecosystem and facilitating the emergence of new strain resistant to pesticides, accomplished of overcoming plant resistance.

Table 2: Disease severity % of twenty-seven *C. beticola* isolates on susceptible sugar beet cultivar (Polylias cv) to Cercospora leaf spot disease.

Governorates	Isolates	Disease incidence (D.I.) %	Disease severity (D.S.) %
Kafrelshiekh	1	14.500	10.333
	2	16.000	12.666
	3	12.250	9.333
	4	13.500	11.666
	5	12.750	10.666
	6	14.250	13.333
	7	12.500	10.000
	8	13.750	11.666
	9	9.500	7.333
Dakahlia	10	14.250	11.333
	11	12.500	9.666
	12	15.000	11.333
	13	12.000	8.666
	14	9.750	7.666
	15	13.500	10.666
	16	13.250	9.333
	17	16.250	13.333
	18	16.500	14.666
Behira	19	9.750	7.333
	20	11.500	8.333
	21	15.000	12.666
	22	15.500	13.666
	23	14.250	11.333
	24	15.000	14.333
	25	10.500	7.333
	26	10.500	9.666
	27	16.250	13.333
	$L.S.D_{0.05}$	**0.533**	**0.637**

Molecular characterization and phylogenetic analysis of *Cercospora beticola:*

The use of molecular markers, particularly ITS regions, has proved to be valuable not only in distinguishing individual varieties but also in elucidating the phylogenetic relationships among different fungal species. (White *et al*., 1990). To explore the genetic polymorphism and verify the identification of *Cercospora* isolates, PCR amplification was conducted with the ITS1 and ITS4 primers, resulting in PCR products of approximately 600 bp for each *Cercospora* isolate. According to the online blast of NCBI, the selected isolates from the north delta were recognized as *Cercospora beticola* based on ITS sequence analysis. These isolates were preserved at the National Gene Bank, ARC, Egypt (NGB) and registered on www.NCBI.com under the accession numbers shown in Table (3). The phylogenetic analysis of the four isolates of C*ercospora* (Fig. 1) has proved the existence of genetic variation in the

north delta region of the obtained isolates, which was necessary to be registered on the NCBI database. The sequence alignment of the four isolates was compared with that of *Cercospora* cf. sigesbeckiae (strain PP_2012_071) (Accession No. NKQR01000000) using the Mega11 neighbor-joining tree method (NJ) of Saitou and Nei. The NJ method operates on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the level of divergence between the sequences. The calculated distance values are in parentheses, following the isolate name. *Cercospora cf.* sigesbeckiae was used as an outgroup of *C. beticola*.

Table 3: List of *Cercospora beticola isolates NCBI* accession numbers.

Isolate code	Name	NCBI accession numbers
*C. beticola*_ND_1	*Cercospora beticola*	OQ162292
*C. beticola*_ND_2	*Cercospora beticola*	OQ152530.
*C. beticola*_ND_3	*Cercospora beticola*	OQ152531
*C. beticola*_ND_4	*Cercospora beticola*	OQ152534

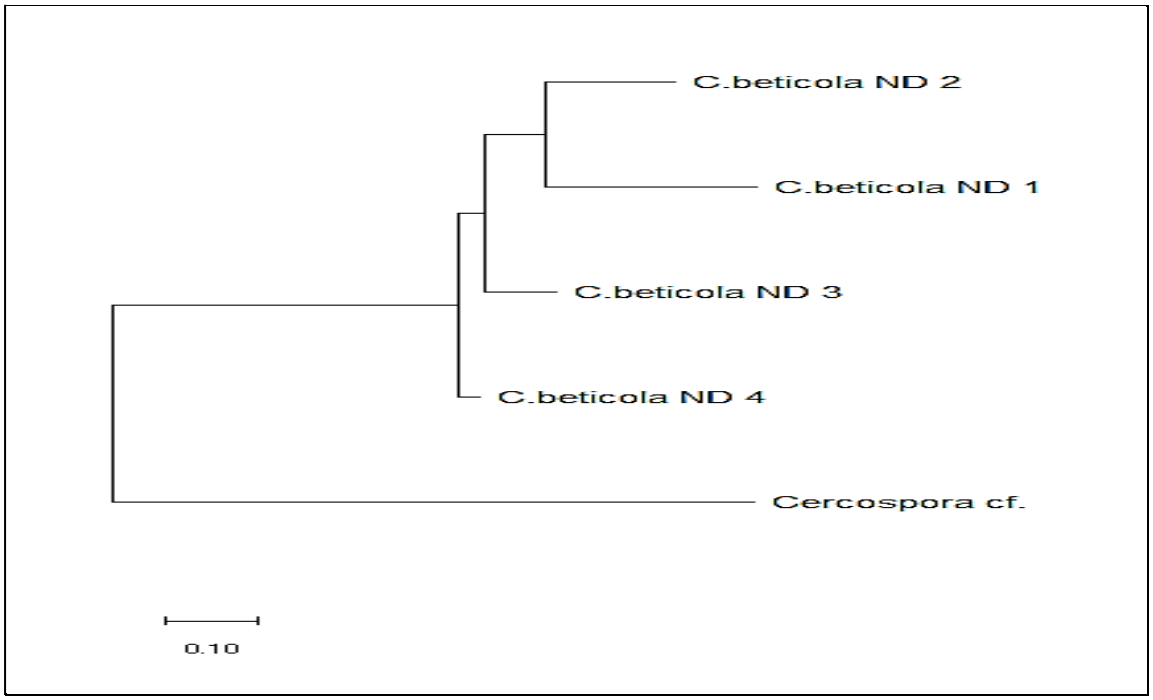

Fig. 1: Phylogenetic assessment of four isolates of *Cercospora beticola* based on nucleotide ITS sequences. The phylogenetic tree was constructed using the Neighbor-Joining method (NJ) of Saitou and Nei through the Mega 11 software.

Impact of treatments on *Cercospora beticola* under laboratory circumstances:

To address the adverse consequences associated with chemical pesticides, scientists are actively exploring alternative methods. This has spurred the investigation of options beyond chemical pesticides, such as essential oils, including Citronella (*Cymbopogon citratus*) and Rose Geranium (*Pelargonium graveolens*) (Mahmoudi *et al*., 2018; Balahbib *et al*., 2020; Elouadi *et al*., 2022; María *et al*., 2022). In our study, two concentrations of essential oils *Pelargonium graveolens* and *Cymbopogon citratus* (five and ten ml per liter) were evaluated. Results shown in Table (4) illustrate that the essential oils Citronella and Rose geranium were most effective in reducing the linear growth (cm) of *Cercospora beticola* compared with the control treatment. The essential oil, rose geranium, had the highest reduction in linear growth %, that is, 85.93 and 89.06 % at 5ml /L and 10ml/ L, respectively. followed by essential oil

Citronella which recorded 76.56 and 82.81 reduction of linear growth % at 5 ml /L and 10 ml/ L, respectively. On the other hand, the fungicide Montoro 30% recorded 92.18 reduction in linear growth %. These findings align with those reported by Mark Angelo *et al*. (2019), who demonstrated that essential oils from *Cymbopogon* species possess antifungal properties and can inhibit the activity of various pathogens such as *Fusarium oxysporum, C. gloeosporioides, C. falcatum,* and *Neopestalotiopsis* spp. This highlights the broad spectrum of pathogens for which citronella essential oil may be effective in managing plant diseases. Specifically, in *C. gloeosporioides*, citronella essential oil hurt germ tube elongation, underscoring its significant influence on the reproduction of *C. gloeosporioides*.

Table 4: Efficacy of two essential oils (Citronella and Rose geranium) on reduction of linear growth % of *Cercospora beticola* under laboratory test.

No.	Treatment	Conc.	Mean of linear growth (cm)	Reduction of linear growth %
1	Untreated (Control)	Water	6.4	00.00
2	Citronella *(Cymbopogon citratus)*	5ml/ L	1.5	76.56
		10 ml/ L	1.1	82.81
3	Rose geranium *(Pelargonium graveolens)*	5ml/ L	0.9	85.93
		10 ml/ L	0.7	89.06
4	Montoro 30%	1 ml/ L	0.5	92.18
$L.S.D_{0.05}$				1.654

Efficacy of essential oils against *C. beticola* under field circumstances:

The impact of the two treatments involving essential oils, namely Citronella at a concentration of 10 ml/L, *Pelargonium graveolens* at a concentration of 10 ml/L, and the fungicide Montoro 30% at a concentration of 1 ml/L, as compared to a control treatment using water. The severity of the CLS disease was assessed 24 hours after each of the three sprays in two consecutive seasons (2020/2021 and 2021/2022), as shown in Tables (5 and 6). The results indicated a significant reduction in the severity of *C. beticola* due to the application of the mentioned treatments compared to the control treatment in both seasons. The tested treatment, *Pelargonium graveolens* essential oil at 10 ml/L, was the most effective against *C. beticola* followed by Citronella**,** essential oil at 10 ml/L in the first and second seasons, then they reduced D.S. % by 61.666 and 53.333% after the first spray, 66.923 and 56.538% after the second spray, and 70.000 and 65.238% after the third spray in the first season, respectively. While they produced a decrease of disease severity by 73.529 and 51.470 after the first spray, by 60.000% and 51.666% after the second spray, and by 68.779 and 61.737% after third spray in the second season, respectively, these results indicated the essential oil *Pelargonium graveolens* at 10 ml/L was effective in reducing infection by CLS causal organisms at all tested sprayings, followed by Citronella essential oil at 10 ml/L during the first and second tested seasons. In general, these vital oils have the potential to aid in the prevention of diseases and exhibit non-toxic effects on plants. Moreover, they can enhance, utilize, and advocate for a comparatively secure, eco-friendly, and cost-effective approach to disease management. These findings align with those reported by Chen *et al*. (2014), who found that citronella oil effectively inhibits *A. alternata* both in vitro and in vivo. Thus, it had been considered a promising natural product for controlling black rot in cherry tomatoes. Scanning electron microscopy revealed an abnormal mycelial morphology. Elouadi *et al*. (2022), further noted that *Pelargonium graveolens* essential oil served as an effective antifungal agent against *Botrytis cinerea, Penicillium expansum,* and *Rhizopus stolonifer*. Furthermore, it is noteworthy that essential oils are recognized for their attributes of low toxicity, biodegradability, and lack of persistence in the environment. Considering these advantages, essential oils have the potential to be used as

alternatives to synthetic fungicides to protect plants from phytopathogenic fungi and prevent the spoilage of food products during storage.

Table 5: Disease severity and effectiveness of various treatments against *Cercospora beticola* in the first season.

No.	Treatments	% Of reduction of CLS disease severity after:						Mean of Reduction %
		1st spray		2nd spray		3rd spray		
		D.S. %	Reduction %	D.S.%	Reduction %	D.S.%	Reduction %	
1	Citronella	5.666	53.333	11.333	56.538	14.666	65.238	58.369
2	*Pelargonium graveolens*	4.666	61.666	8.666	66.923	12.666	70.000	66.196
3	Mentoro30%	1.666	86.666	7.000	73.076	7.666	81.904	80.548
4	Untreated (Control)	12.000	0.000	26.000	0.000	42.000	0.000	-
L.S.D$_{0.05}$		2.075	4.632	3.398	3.897	3.059	2.999	3.111

Table 6: Disease severity and effectiveness of various treatments against *Cercospora beticola* in the second season.

No.	Treatments	% Of reduction of CLS disease severity after:						Mean of Reduction %
		1st spray		2nd spray		3rd spray		
		D.S.%	Reduction %	D.S.%	Reduction %	D.S.%	Reduction %	
1	Citronella 10ml/L	6.666	51.470	11.666	51.666	16.333	61.737	54.957
2	*Pelargonium graveolens* 10ml/L	3.666	73.529	9.666	60.000	13.333	68.779	67.436
3	Mentoro30%	2.000	85.294	6.666	72.500	9.333	78.169	78.654
4	Untreated (Control)	13.666	0.000	24.000	0.000	42.666	0.000	0.000
L.S.D$_{0.05}$		1.529	4.985	3.608	4.996	2.620	3.996	3.053

The impact of various treatments on the qualities of sugar beet root content was examined by estimating the total soluble solids and sucrose percentage. The findings presented in Table (7) and Fig. (2) indicate that all tested treatments resulted in a significant increase in both total soluble solids content and sucrose % compared to the control during the two growing seasons. Among the treatments, the most effective in enhancing total soluble solids content and sucrose content was the essential oil *Pelargonium graveolens* at a concentration of 10 ml/L. It achieved a remarkable increase of 24.00 and 24,500% and 19.20 and 19.60% in the 1st and 2nd seasons, respectively. Following closely was the essential oil *Cymbopogon citratus*, which recorded 22.00 and 23.00% and 17.60 and 18.40% in the 1st and 2nd seasons, respectively. In comparison, the control treatment exhibited values of 18.00 and 18.50% and 14.40 and 14.80% in the 1st and 2nd seasons, respectively. This aligns with the findings of Fatouh *et al*. (2011), who demonstrated that a concentration of 5.0 ml/L of essential oil citral led to a 6.7% increase in total soluble solids (TSS) in sugar beet yield. Additionally, a slight increase in TSS was observed with Citral at 2.5 ml/L, methyl anthranate, and Nerol at 5.0 ml/L for each treatment. They indicated that essential oils served as environmentally friendly natural compounds for managing Cercospora and Alternaria leaf spot diseases in sugar beet plants under field conditions.

Table 7: The impact of the experimented treatments on the crop production, overall concentration of soluble solids (TSS)% and sucrose% in sugar beet plants.

No.	Treatment	TSS %		Sucrose %	
		1st season	2nd season	1st season	2nd season
1	*Citronella*	22.00	23.00	17.60	18.40
2	*Pelargonium graveolens*	24.00	24.50	19.20	19.60
3	Mentoro30%	20.00	21.00	16.00	16.80
4	Untreated (Control)	18.00	18.50	14.40	14.80
L.S.D$_{0.05}$		0.954	1.002	0.991	0.973

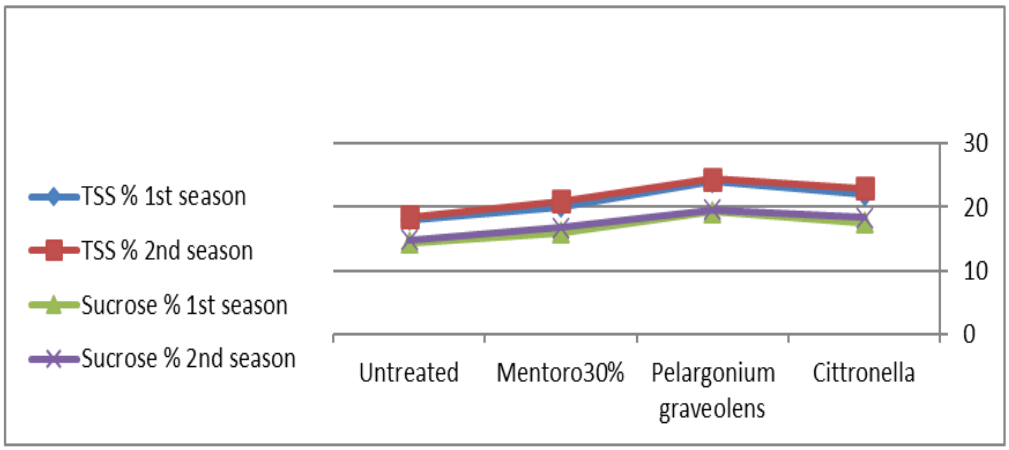

Fig. 2: Impact of the applied treatments on TSS% and Sucrose % of sugar beet.

One of the key criteria examined to assess the positive impact of the treatments on controlling plant pathogens is their effect on the activity of oxidative enzymes (Swelum *et al*., 2020). Therefore, the activities of oxidative enzymes, including polyphenol oxidase and peroxidase, have been evaluated, and their roles in inducing plant resistance against pathogens have been established. Polyphenol oxidase and peroxidase, two enzymes of utmost importance, were evaluated in this study to determine the impact of the materials used on their activity. The results, presented in Tables (8 and 9), demonstrate a consistent pattern. The activity of these enzymes exhibited a gradual increase when sugar beet plants were treated with the tested materials. Among all the treatments, the essential oil *Pelargonium graveolens* at a concentration of 10 ml/L showed the most positive effect on enhancing the activity of both polyphenol oxidase and peroxidase enzymes. For instance, the activity of the peroxidase enzyme increased from 1.055 to 1.345 within the first 5 minutes after the second spray and from 1.324 to 1.665 within the same time frame after the third spray, following six days of treatment. Followed by the essential oil Citronella (*Cymbopogon citratus*) at 10 ml/L, while increasing the activity of pox from 0.950 to 1.255 within 0.00 min to 5.00min after six days of the second spray and from 1.285 to 1.627 within 0.00 min to 5.00 min after six days of the third spray. The pesticide Mentoro 30% at 1 ml/L recorded the lowest effect in the increased enzyme activities, increasing pox activity from 0.876 to 1.196 within 0.00 min to 5.00 min after six days of the second spray and from 1.269 to 1.584 within 0.00 min to 5.00 min after six days of the third spray. The same trend was observed in the case of polyphenol oxidase (PPO) enzyme activity.

These findings further support the effectiveness of these essential oils in reducing the severity of the pathogen. These findings align with those of Pereira *et al*. (2012), who reported that citronella essential oil partially controls rust and brown eye spot in coffee plants and enhances the plant defense system. This led to significant increases in the activities of POX and CHI enzymes, as well as the accumulation of lignin in coffee leaves 336 h after spraying. Furthermore, Sehsah *et al*. (2022) and Hamden *et al*. (2023) noted that treating sugar beet plants

with specific bioagents and natural substances led to a significant enhancement in the activity of polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase against CLS disease in sugar beets. These findings highlight the potential of environmentally friendly chemicals used in the treatments, which can boost the plant's enzymatic defense system. This, in turn, can be effective in combating *C. beticola* in vitro and controlling Cercospora leaf spots *in vivo* while also reducing the severity of the pathogen.

Table 8: The impact of the essential oils Citronella and *Pelargonium graveolens* on the activity of the peroxidase (POX) enzyme.

Treatment	Peroxidase (POX)											
	Six days after the second spray						Six days after the third spray					
	0.00 min	1.00min	2.00min	3.00min	4.00min	5.00min	0.00 min	1.00min	2.00min	3.00min	4.00min	5.00min
Citronella	0.950	1.043	1.127	1.178	1.225	1.255	1.285	1.396	1.506	1.553	1.612	1.627
Pelargonium graveolens	1.055	1.132	1.220	1.295	1.330	1.345	1.324	1.460	1.553	1.610	1.654	1.665
Mentoro	0.876	0.985	1.062	1.097	1.137	1.196	1.269	1.349	1.465	1.498	1.563	1.584
Untreated (Control)	0.863	0.944	1.004	1.070	1.098	1.167	1.220	1.287	1.321	1.396	1.435	1.482
L.S.D$_{0.05}$	0.043	0.021	0.025	0.011	0.013	0.015	0.012	0.014	0.011	0.045	0.034	0.042

Table 9: The impact of the essential oils Citronella and *Pelargonium graveolens* on the activity of the polyphenol oxidase (PPO).

Treatment	Polyphenol oxidase (PPO)											
	Six days after the second spray						Six days after the third spray					
	0.00 min	1.00 min	2.00 min	3.00 min	4.00 min	5.00 min	0.00 min	1.00 min	2.00 min	3.00 min	4.00 min	5.00 min
citronella	0.176	0.245	0.318	0.359	0.384	0.397	0.408	0.442	0.485	0.502	0.527	0.539
Pelargonium graveolens	0.235	0.289	0.338	0.381	0.396	0.428	0.458	0.488	0.508	0.533	0.568	0.585
Mentoro30%EC	0.125	0.196	0.223	0.283	0.328	0.375	0.404	0.431	0.459	0.478	0.491	0.503
Untreated (Control)	0.103	0.149	0.186	0.219	0.249	0.276	0.395	0.416	0.439	0.463	0.479	0.484
L.S.D$_{0.05}$	0.023	0.014	0.015	0.011	0.012	0.021	0.018	0.013	0.010	0.021	0.024	0.016

Analysis using scanning electron microscopy (SEM):

Scanning electron microscope (SEM) was conducted to investigate how these treatments affect the growth of the fungus responsible for Cercospora spot disease, the formation of conidiophores and conidiospores, and the size of infection spots on beet leaves. SEM is a valuable tool for examining fungal growth and the growth of other organisms on plant surfaces (Jackowiak *et al*., 2005). The analysis of the scanning electron microscope slides demonstrated that the application of essential oils Citronella and *Pelargonium graveolens*, along with the fungicide montoro 30%, resulted in a decrease in the number of spots on the beet leaves. Additionally, the slides revealed the destruction and deterioration of fungus conidiophores and conidiospores, as well as the obstruction of most stomata in the affected regions. These observations were depicted in Figs. (3 and 4), show casing varying degrees of blockage. Furthermore, the tests indicated the inability to form conidiophores and conidiospores (Fig. 5), while simultaneously displaying their distorted appearance. The slides also confirmed the destruction and degradation of fungus conidiophores and conidiospores (Figs. 3, 4 and 5), along with the presence of stomatal blockage in the affected areas. This outcome aligns with

previous studies on plant pathogenic fungi, where substances such as *B. subtilis* and certain plant extracts were found to have similar effects on *C. beticola* due to their fungal inhibitory properties (Sehsah *et al*., 2022). Furthermore, scanning electron microscopy revealed significant abnormalities in mycelial morphology. *Cymbopogon citratus* has been found to possess antifungal properties, inhibiting mycelial growth and spore germination of *Colletotrichum gloeosporioides* from mangoes (Rabari *et al.,* 2018) and *Fusarium oxysporum* f. sp. *lycopersici* (Sharma *et al*., 2018).

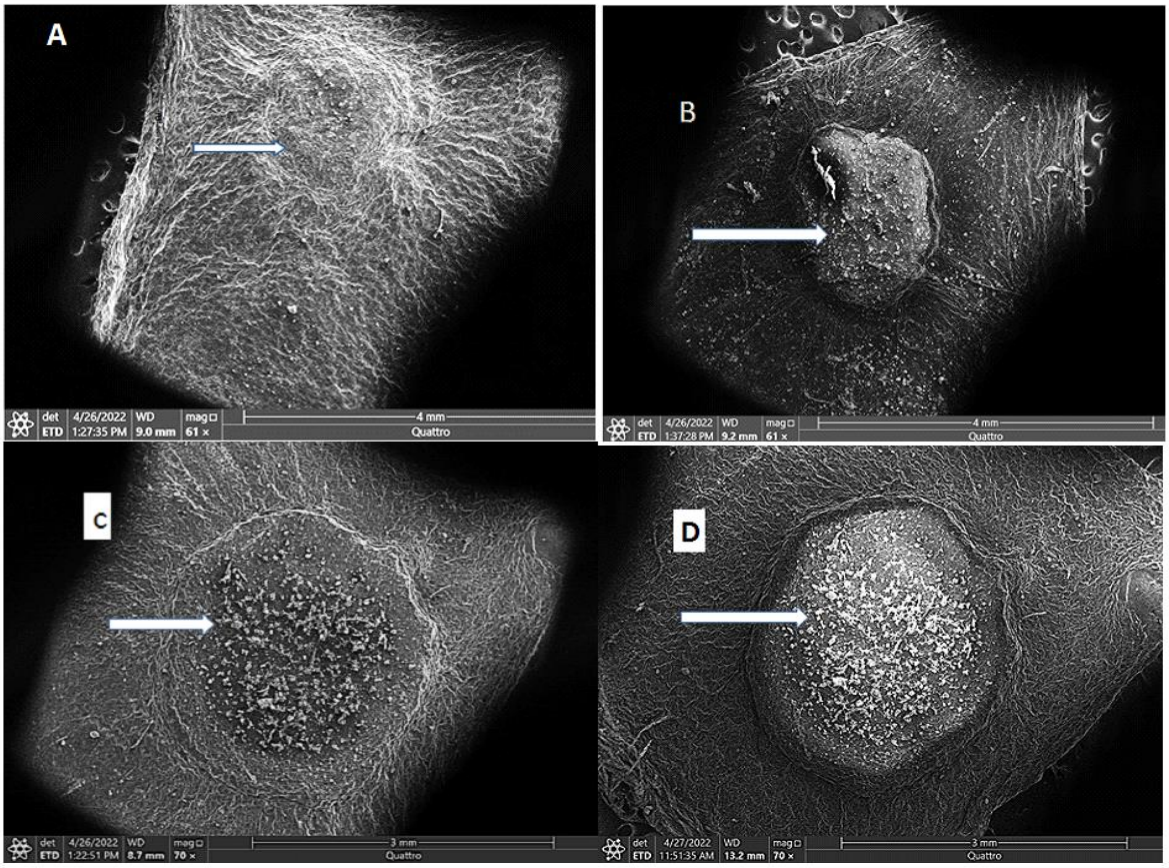

Fig. 3: A: The usual and standard appearance of Cercospora spots, displaying a high abundance of conidiophores and conidiospores (control treatment). B (Montoro 30%), C (Citronella), and D (*Pelargonium graveolens*): The treatments' impact is evident as they inflicted damage and resulted in a significant decrease in the surrounding area and a noticeable reduction in the quantity of reproductive structures of the fungus, potentially leading to its complete disappearance in a single spore

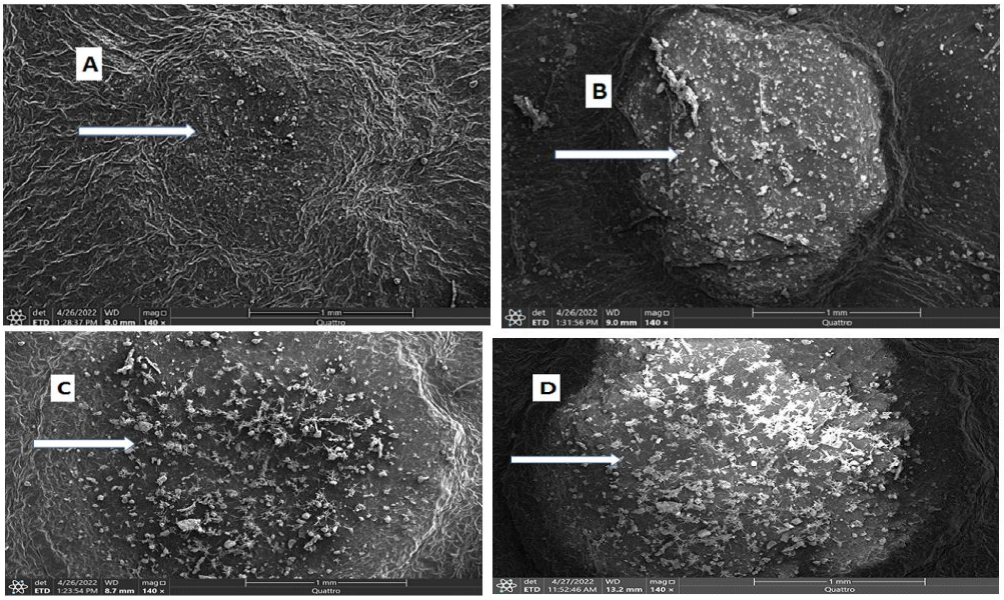

Fig. 4: A: The ongoing presence of the usual and characteristic appearance of Cercospora spots, exhibiting a high abundance of conidiophores and conidiospores. Additionally, there is a distinct boundary and a significant transparent halo observed between the affected and healthy tissue (control treatment). B (Montoro 30%), C (Citronella), and D (*Pelargonium graveolens*): The impact of these treatments is evident as they induce escalating harm and a substantial reduction in the aura, along with a noticeable decrease in the quantity of reproductive structures produced by the fungus

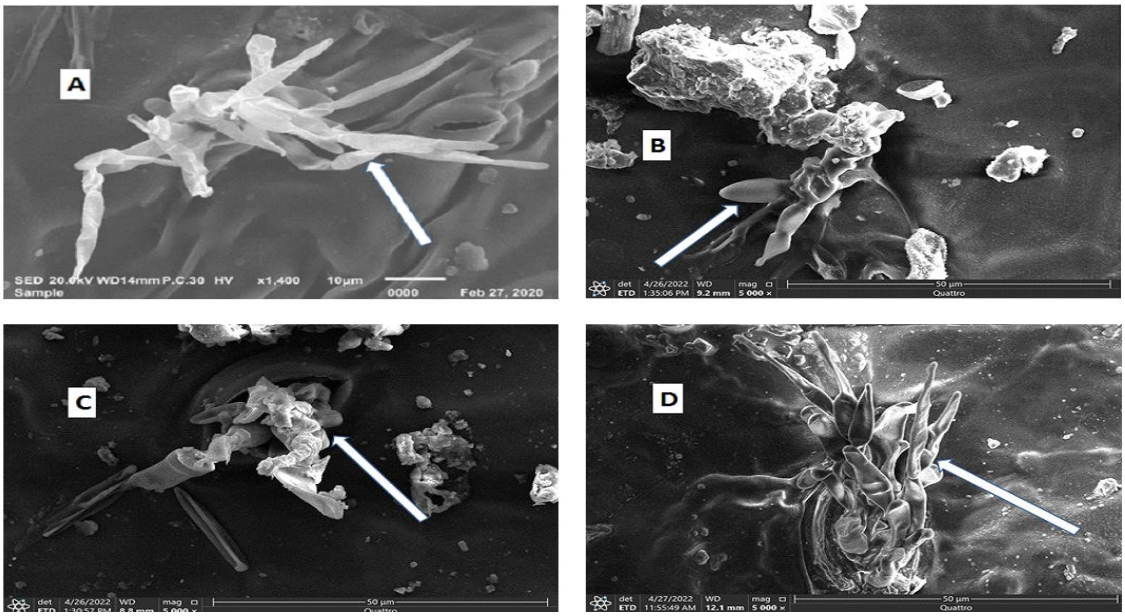

Fig. 5: The application of various essential oils treatments on plants has resulted in the degradation of spores and conidiophores B (Montoro 30%), C (Citronella), and D (*Pelargonium graveolens*), as observed through a simple comparison with the control (A). Consequently, the fungus lost its viability, rendering it incapable of initiating new infections.

Conclusions:

This study reveals the efficacy of essential oils, Citronella (*Cymbopogon citratus*) and Rose geranium (*Pelargonium graveolens*) in the management of Cercospora leaf spot disease. The application of these essential oils at a concentration of 10ml/L significantly improved the resistance to infection by *C. beticola*. These treatments not only increased the overall sugar content and sucrose percentage in the sugar beet yield but also enhanced the activation of antioxidant enzymes (POX and PPO). Additionally, these treatments led to dehydration and

breakdown of the fungal structures (conidiophores and conidiospores) of *C. beticola*. Consequently, they can serve as environmentally friendly alternatives to commercial fungicides and offer cost-effective solutions for disease control. However, further research is required to determine the chemical composition of the essential oils used in this study and elucidate their molecular mechanisms during infection.

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