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Biological control of *Ziziphus spina-christi* leaf spots and using the leaf extract against some plant diseases

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ABSTRACT

The dominant Sidr variety in Siwa Oasis (thorny) was less affected than the thornless variety. A number of leaf spot-causing fungi were isolated: *Alternaria alternata*, *Cercospora zizyphi*, *Cladosporium gloeosporioides* and *Fusarium semitictum*. *Alternaria* isolates were the most frequent, followed by *Cercospora* isolates, while *Fusarium* and *Cladosporium* isolates were the least frequent. All isolates grew successfully on all tested heavy metal salt concentrations (copper sulfate, zinc phosphate, silver nitrate). *Alternaria* isolates were the most tolerant, followed by *Cercospora* isolates, while the growth of *Fusarium* and *Colletotrichum* isolates was reduced but not stopped. Aqueous extract from Sidr leaves, at various concentrations, successfully suppressed the growth of the tested fungi. *Fusarium oxsporum*, followed by *Fusarium solani*, and then *Rhizoctonia solani* were the most inhibiting, while *Fusarium*, *Aspergillus* and *Rhizopus* are more tolerant. *Ziziphus* leaf extract showed its effectiveness in reducing soft rot of citrus fruits after storage at room temperature for 7 days. The rot was reduced by 68.9% with the original extract concentration, 50.3% with 50% concentration, and 39.2% with 25% concentration.

Keywords: Ziziphus leaf spot, *A. alternata*, *C.gleosporioides*, *F. semitictum*, *C.ziziphi* , Sidr leaf extract as antifungal , Spent mushroom substrate as antifungal , Penicilliun mold control.

INTRODUCTION

Chemical analyses of Sidr leaves have indicated that they contain numerous of antioxidant and antimicrobial compounds, such as alkaloids, tannins, steroids, flavonoids, and saponins (Sarjito *et al.*, 2021). Abdulrahman *et al*., 2022, reported that polyphenols and flavonoids were the most common compounds, comprising 66 compounds out of a total of 193 reported compounds from different parts of the plant. Traditional medicinal practices have long demonstrated the benefits of these leaves in treating many human diseases, such as skin infections such as scabies, pimples, and dandruff (Al-Mutarii *et al.*, 2016), and killing intestinal worms (El-Kutry *et al*., 2020). Recently, they have been used to control fish diseases in farms (Temerk *et al*., 2017), and in raising the level of saliva. Human immunity is enhanced when added as a powder in pizza dough (Falciano *et al.*, 2022) and in children's biscuits (El-Seedy *et al*., 2021). The extract also has a strong protective role against aflatoxin poisoning produced by the fungus *Aspergillus flavus* (Abdel-Wahhab *et al.*, 2007). It has also been proven that a mixture of olive leaf and jujube leaf extract produces a yellowish-brown

color on cotton with good stability to light, washing, and perspiration, making it a source of environmentally friendly natural dyes for dyeing cotton. It is known that Sidr trees (Rhamnaceae – *Ziziphus* spp.) grow and spread naturally in tropical and subtropical desert regions of the world (Orwa *et al*., 2009). These are economically fragile areas, which doubles the environmental importance of these trees, which grow in dense clusters, making them effective in combating desertification (Bedair *et al*., 2020). The Egyptian Siwa Oasis, located near the Mediterranean coast and adjacent to the Libyan Desert, is one of these areas where Sidr trees of the *Ziziphus spina-christi* species are naturally widespread, and whose trees were selected for examination in this study (Abdelghani and Fawzy, 2006). There is a lot of research in Egypt on Sidr trees from the botanical or pharmaceutical point of view, but pathological studies on them are rare (Shehata *et al*., 2022). The current study aimed to identify the fungi that attack Sidr leaves and reduce their vitality in an attempt to combat them. It also attempts to shed light on the inhibitory effect of the extract of these leaves on the growth of plant-pathogenic fungi. It is known that there are many studies that indicate the efficiency of Sidr leaves extract in inhibiting the growth of bacterial cells (Gheith and El-Mahmoudy, 2018 and Sarjito *et al*., 2021), but only a few of them indicate its antifungal effect on the growth of fungi, whether they are pathogenic to humans, such as Candida yeast (Hsu *et al*., 2021), or dermatophytes, such as Trichophyton (Yassin and Sgheer, 2024), or opportunistic fungi that attack the respiratory system, such as *Aspergillus fumigates* (Saddiq, Amna, 2014), or pathogenic for plants such as *Alternaria citri* (El-Shahir *et al*., 2022). It is well known that citrus fruits have a relatively short post-harvest lifespan, and losses are increasing in developing countries such as Egypt and India due to unscientific practices in picking, packaging, transportation, and storage (Sonkar *et al.*, 2008). Therefore, it is crucial to develop post-harvest treatments for Citrus fruits to reduce losses. Blue green soft rot is the most common post-harvest disease of Citrus fruits, causing an estimated 30–50% of total production loss. There is a need to replace traditional fungicides with environmentally friendly ones (Ismail & Zhang, 2004).

MATERIAL AND METHODS

Collection of infected Sidr leaves:

Infected leaves were collected from the common Sidr variety in Siwa Oasis (Evergreen, thorny) and other infected leaves were also collected from less common Sidr trees (Deciduous, thornless). Polyethylene bags were used to store the collected samples and refrigerated at 4°C to isolate and identify the associated fungal pathogens.

Isolation of the pathogens of Sidr leaf spot:

The infected Sidr leaves were washed 3 times with sterile distilled water and cut with a sterile blade into small pieces (5 mm). The dissected parts were superficially sterilized with sodium hypochlorite (2% solution) for 2-3 minutes and then re-washed several times with sterilized distilled water. The superficially sterilized samples were dried with dry, clean, sterile paper towels to remove excess water. Fungal pathogens were isolated from the infected leaves by following tissue culture methods according to Aneja (2003). The sterile pieces were placed onto sterile PDA plates and incubated upside down at room temperature (25 ± 2 °C). Starting from the second day of incubation the concerned plates were monitored for a week to monitor fungal growth or spore formation.

Purification and identification of the isolated fungi:

The isolated fungi were purified by culturing individual spores and/or culturing the tip of fungal hyphae according to (Aneja, 2003), the pure fungal isolates were examined and their morphological characteristics were recorded, then the fungi were identified by plant

pathologists at Al-Azhar Faculty of Agriculture through the growth characteristics of the fungal isolates on PDA plates and their spores under the microscope and compared with the available references and standards (Barnett and Hunter, 1972) and (Sutton, 1980).

Testing the ability of the isolated fungi to grow in a medium containing some heavy metal salts:

The isolated fungi, *i.e. Alternaria alternata, Cercospora zizyphi, Cladosporium gloeosporioides* and *Fusarium semitictum* were tested for their tolerance to different concentrations of some heavy metals. Potato dextrose agar medium was used to test the resistance to heavy metals. Different concentrations (0, 250, 500, and 1000 mg/L) of Pb $(NO_3)_2$, Cu SO_4 and $Zn(PO_3)_2$ were used for fungal selection. Incubation was carried out at 25 °C for one week, growth was monitored by measuring the growth from the inoculation point at the center of the colony. Three replicates were taken for each treatment. Fungal tolerance was studied by determining the minimum inhibitory concentration (MIC) (Iram *et al*., 2013).

Extracting of spent mushroom substrate:

The compost used in this experiment was spent substrate from commercial mushroom production (*Agaricus bisporus*) provided by the Mushroom Production Unit, Faculty of Agriculture, Al-Azhar University, the substrate was prepared according to standard protocols (Cotter, 2014), inoculated with commercial mushroom isolate, placed in a peat cover, and kept in production for 3 to 4 months. The spent substrate was removed without any further treatment during January 2024 in 2 m^3 batches and stored without stirring, the fertilizer quantities were mixed with water (1:2 spent substrate weight/water volume) in plastic containers and incubated without stirring in the laboratory at 15-25°C for 7-8 days. After incubation, the contents of the container were stirred and SMS extracts were obtained by filtration through a single layer of cheesecloth. This filtration process resulted in maximum particle sizes ranging from 100 to 125 µm in diameter.

Effect of spent mushroom substrate extract on growth of the fungi isolated from Sidr leaves:

The poisoned food technique (**Erhonyota** *et al*., 2023) was used to test the effect of SMC on the growth of Sidr leaf pathogenic fungi, *i.e. A. alternata*, *C. ziziphi*, *C. gloeosporioides*, *F. semitictum in vitro* under laboratory conditions. To prepare aqueous extracts of SMC, 5, 10 and 15 g of the prepared SMC were suspended in 100 ml of sterile distilled water to obtain concentrations of 5, 10 and 15%, respectively. Each suspension was mixed thoroughly to evenly distribute the particles. For each concentration, two batches were made: one was sterilized via steam autoclaving at 121 °C for 15minutes, and the other was left unsterilized. Then,1ml from each batch (sterile and non-sterile) of the varying SMC concentrations was dispensed into sterile Petri dishes. After wards, 10ml of cooled, liquid PDA was poured aseptically into each dish and swirled softly to mix the SMC with the agar. Control plateshad1 ml of sterile distilled water mixed with cooled, molten PDA. The plates were allowed to solidify. To start the experiment, a 5 mm fungal disc was taken from the edge of a5-day-oldfungalcultureofthetargetpathogen and placed in the middle of the plates. All plates were then kept at 25 ± 2 °C. Data regarding fungal growth was gathered and noted, beginning three days after inoculation, until the control plates showed complete growth of the pathogens. There were three replicates for each test. The percentage inhibition in fungal growth was determined using the formula below:

$$I = \{ (C - T)/ C\}100$$

where I = Percentage inhibition in fungal growth, C = Radial growth of the pathogens in the control.

Preparation of aqueous extract of Sidr leaves:

Healthy leaves were collected from naturally growing Sidr trees *Z. spina-christi* in Siwa Oasis, washed and left to dry, After washing and drying leaves were grinding and 100 side leaves powder was added to 200ml of sterilized distilled water or ethanol, and after 24 hours of soaking at room temperature, both the aqueous and alcoholic extracts were filtered using three layers of Whatman No. 1 filter paper , then subjected to a centrifugal force of 3000 rpm for 15 minutes. The clear liquid was collected, the concentrations used in this research were 25%, 50% and 100%, the extracts were sterilized using a membrane filter with a diameter of 0.45 µm and stored in dark cups in the refrigerator until use (Adzu *et al*., 2001).

Effect of the aqueous extract of Sidr leaves on growth of some plant pathogenic fungi:

Five isolates of plant pathogenic fungi were obtained from MIRCEN Center, Faculty of Agriculture, Ain Shams University, Egypt. They were incubated at 25°C. A suspension of experimental fungal spores was prepared after growing them for 10 days on PDA medium, by adding 5 ml of sterile distilled water to the growth. The poisoned food technique was used to test the effect of aqueous extract of Sidr leaves on the growth of plant pathogenic fungal isolates *in vitro* under laboratory conditions according to (Akhtar *et al.*, 2025). Extract concentrations (25%, 50%, and 100%) were prepared as previously described. One ml of each different concentration was poured onto sterile Petri dishes. Then, ten milliliters of melted, cooled PDA was poured aseptically into each dish and swirled to spread the extract evenly within the agar. Control plates, using sterile distilled water mixed with the cooled PDA (0 g extract/ml), were also prepared. The plates were allowed to harden. A5 mm diameter fungal disc, taken from the margin of a fresh (five-day-old) fungal pathogen culture, was placed in the center of each plate for inoculation. Each treatment was performed in triplicate. All plates were incubated at 25 ± 2 °C. Fungal growth measurements were taken, starting three days post-inoculation and continuing until the control plates were entirely covered. The growth of the pathogens was then assessed. The percentage inhibition of fungal growth (in millimeters) was calculated using the provided formula.

Inhibition percentage= A – B /A × 100

Where:

A= mean diameter of growth in the control treatment.

B= mean diameter of growth in a given treatment.

Effect of Sidr leaf extract on postharvest Citrus Penicillium rot:

Mature Valencia orange fruits were harvested from the garden of the Agricultural Research Center at Giza in February 2024, and were immediately transferred to the Plant Pathology Laboratory at the Faculty of Agriculture, Al-Azhar University. The fruits were selected with uniform size and color, free from wounds and mechanical injuries. The fruits were washed with tap water and then dried. The surface of the fruits was disinfected by immersing them in a solution of sodium hypochlorite 1 g × 100 ml^{-1} for 5 minutes. Then, they were washed with distilled water. Postharvest experiments were conducted to evaluate the effect of Sidr leaf extract in protecting orange fruits from Penicillium mold infection. The experiment consisted of 4 treatments, with 10 orange fruits per treatment: (1) control without fungus and without Sidr extract; (2) control without fungus and with Sidr extract; (3) Pathogenic fungus without Sidr extract, (4) Pathogenic fungus with Sidr extract. In treatments, orange fruits were wounded in two equatorial positions with the help of a sterile needle to a depth of 1.5 mm and a width of 2.0 mm, and a suspension of the mold-causing fungus (*Penicillium digitatum* or *P. italicum*) (10^8 spores/ml) was prepared from a 7-day-old colony on PDA medium, 10 µl of the fungal spore suspension was added over each wound.

The fruits were immersed in the aqueous extract of Sidr leaves at concentrations of 25% or 50% or 100% for 5 min, the fruits were kept at room temperature (21°C) and ambient air (50% relative humidity), with a photoperiod of 12 h for 7 days . Another number of healthy, unwounded orange fruits were also immersed in sterile distilled water and kept in the same room as a control treatment. Three replicates were taken for each treatment. For assessment of percent rot of the fruits in inoculation and Sidr extract evaluation experiments, the modified disease fruit grading standard was as following according to (Yu, 2010):

Grade 0: healthy; Grade 1: mold area ≤ 20%; Grade 2: mold area >20 and ≤ 40%; Grade 3: mold area >40 and ≤ 60%; Grade 4: mold area > 60 and ≤ 80%; Grade 5: mold area > 80%.

Ɛ(Number of fruit diseased X number of grade) / Total number of fruits X Highest number of grade.

The efficacy of the different treatments was calculated according to the Disease Reduction Index (DRI) formula as suggested by (Gutter, 1969):

DRI = (Percent rot in control - Percent rot in treatment) / (Percent rot in control) × 100

Statistical analyses:

Analysis of experimental data was achieved by using one-way analysis of variance (ANOVA), while means differences were separated using Duncan's multiple range test and the (LSD) at 5.0% level of probability following Costate Software (Snedecor & Cochran, 1982).

RESULTS AND DISCUSSION

Isolation and identification of the pathogens of Sidr leaf spot:

Seventy-four fungal isolates were isolated from the leaves of Siwa Oasis Sidr trees (Table 1). The dominant Sidr variety (thorny) in the Oasis was less infected (26 isolates) compared to the thornless variety (48 isolates).

Table (1): Fungal isolation from leaves of two Sidr varieties at Siwa Oasis during January 2024.

Fungal isolate	Isolation from leaves of two Sidr varieties		
	Thorny	Thornless	Total number
Alternaria alternate	11	20	31
Cercospora zizyphi	08	13	21
Cladosporium gloeosporioides	04	10	14
Fusarium semitictum	03	05	08
Total number	26	48	74

Most of the spots appeared on the lower leaves, and the leaves bore circular brown to gray spots usually surrounded by a yellow halo. Pure isolates were defined by the research team in the Department of Botany - Division of Plant Pathology, Faculty of Agriculture, Al-Azhar University, as follows:

Alternaria alternata, Cercospora zizyphi, Cladosporium gloeosporioides and *Fusarium semitictum.* The genus *Alternaria* was the most isolated fungus, followed by Cercospora, while Fusarium and Cladosporium were the least isolated fungus.

This result is consistent with the findings of Jamadar *et al*., 2009, Hoque *et al*., 2016, and Hawar *et al*., 2022. Most researchers (Bai *et al*., 2015; Li *et al*., 2015 and Abd El-Gaffar, Rasha *et al*., 2022) agree that *Alternaria alternata*, Cladosporium, and Cercospora are the most common fungi causing leaf spotting in Ziziphus trees worldwide. However, Chinese

jujube trees (*Z. jujube*) are unique in being infected with Nothophoma and *Fusarium incarnatum*, as indicated by the results of Guo *et al*., 2015, and Wright *et al*., 2019.

Testing the ability of the isolated fungi to grow in a medium containing some heavy metal salts:

The results of growth of the fungi causing leaf spot of Ziziphus on PDA medium amended with heavy metal salts, *i.e.* Copper sulphate, zinc phosphate and silver nitrate (Table 2) indicate that all the isolates grew successfully on all the tested heavy metal salt concentrations. Alternaria isolates were the most tolerant, followed by Cercospora isolates, while the growth of Fusarium and Colletotrichum isolates was reduced but not stopped.

Table (2): Testing the ability of the isolated fungi to grow in a medium containing some heavy metal salts.

Fungal isolate	Control	$CuSO_4$ Cons. with mg /L			$Zn(Po_4)_2$ Cons. With mg/L			$Pb(NO_3)_2$ Conc. with mg /L		
		250	500	10^3	250	500	10^3	250	500	10^3
A.alternata	90	90	85	83	90	87	82	90	85	87
C.ziziphi	90	85	69	95	86	71	65	87	73	65
C.gloeosporioides	90	90	90	85	90	85	83	90	84	87
F.semitictum	90	87	70	67	87	72	66	88	75	69

These results are consistent with the findings of Verma *et al*., 2016, and Zhao *et al*., 2016, which indicate that *Alternaria alternata* is capable of absorbing high concentrations of copper, silver, and lead salts. Prajapati *et al*. (2024) found that *Cladosporium* is a halophilic fungus. The known tolerance of fungi to high salt concentrations suggests that these fungi could be used as bio-agents for cleaning polluted water in an environmentally friendly manner. Liu *et al*. (2023) indicated that treating fungal biomass used to remove pollutants with saponin-rich solutions, such as Sidr leaf extract, recharges it electrically, thus increasing its absorption efficiency of heavy metals, given that saponins act as a stress-reducing agent.

Effect of spent mushroom substrate extract on growth of the fungi isolated from Sidr leaves:

The growth of the pathogenic fungi of Sidr leaves on PDA medium modified with concentrations of Spent mushroom_substrate extract (Table 3) indicates the efficiency of the extract in inhibiting the growth of the tested fungi, especially the non-sterile concentrations, and that the inhibition rate increases with increasing concentration.

Table (3): Effect of spent mushroom substrate extract on growth of the fungi isolated from Sidr leaves after completion of growth in control dishes.

Fungal isolate	Control growth (mm)	Inhibition growth (%) under sterile SMC* extract concs.(g/ml)			Inhibition growth (%) under not sterile SMC extract concs.(g/ml)		
	0.0	0.05	0.10	0.15	0.05	0.10	0.15
A. alternata	90	77.8	81.1	87.8	83.3	85.6	100
C.ziziphi	90	81.1	83.3	88.9	85.6	88.9	100
C. gloeosporioides	90	83.3	86.7	90.0	88.9	91.1	100
F.semitictum	90	88.9	90.0	92.2	92.2	94.4	100

*spent mushroom concentration

Fusarium, followed by Colletotrichum, was the most affected fungi, while *Alternaria*, followed by Cercospora, was the most tolerant fungi. The chemical analysis of spent mushroom extract (SME) verified that it contains considerable amount of reducing sugars, phenolic compounds and other macro and micro elements (Abd-Elsattar. Hayat, 2023).

This result is consistent with the findings of Ntougias *et al*. (2008) against *Septoria*, Marin *et al*. (2013) against *Didymella*, Ishihara *et al*. (2018) against *Pyricularia*, and Fujita *et al*. (2021) against *Alternaria*. These results are of great importance because the application of inexpensive, environmentally friendly products such as spent mushroom substrate extract to combat pathogens does not harm the environment, does not leave behind long-lasting compounds, and does not lead to the development of resistant microbial strains.

Effect of the aqueous extract of Sidr leaves on growth of some plant pathogenic fungi:

The growth of some plant pathogenic fungi on PDA medium modified with concentrations of aqueous extract of Sidr leaves (Table 4) indicates the efficiency of the extract in inhibiting the growth of all the tested fungi, especially in the presence of concentrations of 50% and 100%, and that the inhibition rate increases with increasing concentration.

Table (4): Effect of Sidr leaf extract on linear growth of some plant pathogenic fungi on PDA medium.

Test fungus	Colony diameter and inhibition % after treated with Sidr leaf extract			
	00 %	25 %	50 %	100 %
Aspergillus flavus	90	90.0	55.3	39.0
Inhibition%	00	00.0	38.6	56.7
F. oxysporum	90	85.5	54.0	20.3
Inhibition%	00	05.0	40.0.	77.4
Fusarium solani	90	87.3	67.3	24.0
Inhibition%	00	03.0	25.2	73.3
Rhizoctonia solani	90	89.3	65.3	29.7
Inhibition%	00	0.78	27.4	67.0
Rhizopus stolonifer	90	80.7	60.0	40.7
Inhibition%	00	10.3	33.3	54.8

Fusarium oxysporum, followed by *Fusarium solani* and *Rhizoctonia solani* were the most affected fungi, as their growth inhibition rate reached 77.4%, 73.3% and 67.0%, respectively, while the two saprophytic fungi *Aspergillus flavus* and *Rhizopus stlonifer* were the most tolerant fungi, as their growth inhibition rate reached 65.7% and 54.8%, respectively. These results are consistent with the laboratory findings of Zomorodian *et al*., 2010 against Trichophyton and Aspergillus; Daneshman *et al*., 2013 against *Candida* and Aspergillus ; Saddiq, Amna *et al*., 2014 against Fusarium, Rhizoctonia and Aspergillus.

Effect of Sidr leaf extract on postharvest Citrus Penicillium rot:

The results of treating orange fruits Sidr leaf extract (Table 5) indicate the effectiveness of all the tested concentrations (25, 50, 100%) in reducing the percentage of fruit infection with soft rot after storing them at room temperature for 7 days. The inhibition effect was increases with increasing the concentration used, as the mold decreased by 68.9% with the original concentration of the extract; by 50.3% with the 50% concentration, and by 39.2% with the 25% concentration.

This result is consistent with the greenhouse findings of Koita *et al*., 2017 against peanut diseases (Cercospora leaf spot and rust): El-Shahir *et al*., 2022 against Alternaria leaf spot of tomato, and Akhtar *et al*., 2025 against charcoal rot of maize (Macrophomina).

Table (5): Effect of Sidr leaf extract on Penicillium rot of Citrus fruits after 7 days store under laboratory conditions.

Grade of disease index	Number of rotted Citrus fruits after treatment with sidr leaf extract concentrations			
	00 %	% 25	50 %	%100
1	00	00	07	20
2	00	13	11	08
3	03	12	10	02
4	09	05	05	00
5	18	00	00	00
Disease %	90.0	54.7	44.7	28.0
Inhibition %	-	39.2	50.3	68.9

This result is consistent with the findings of several researchers who have used non-toxic plant extracts to reduce the severity of soft rot of Citrus fruits, such as the phenolic extract of *Acacia* leaves (Mekbib *et al*., 2007), tea leaf saponins (Hao *et al*., 2010), *Aloe vera* alkaloid (Zapata *et al*., 2013), the alkaloid extract of *Solanum nigrum* leaves (Musto et al., 2014), the flavonoid extract of poplar leaves (Yang *et al*., 2016), and cinnamon aldehyde (Yang *et al*., 2020).

Conclusion

this study has demonstrated the efficacy of Aqueous extract from Sidr leaves, successfully suppressed the growth of *Alternaria alternata*, *Cercospora zizyphi*, *Cladosporium gloeosporioides*, and *Fusarium semitictum*. Also have the ability to reduce the infection with soft rot (Penicillium mold) at room temperature one week. Future research should focus on isolate and identify antifungal compound, to develop new fungicides.

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