Mesopotamia Journal of Agriculture

https://magrj.mosuljournals.com

THE USE OF SOLAR STERILIZATION OF SOIL PREPARED FOR FOREST SEEDLING AND ITS EFFECT IN INHIBITING THE GROWTH OF SOIL FUNGI AND MOLECULAR DIAGNOSTICS BY USING PCR TECHNIQUE FUNGI

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Article information
Article history:
Received: 10/01/2024
Accepted: 26/03/2024
Published: 31/03/2024

Keywords:
Black mulching, PCR, solar sterilization, soil fungi, temperature effect.

DOI:
https://doi.org/10.33899/mja.2024.145974.1345

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ABSTRACT

The main aim of this study to isolate fungal pathogens from non-sterilized soils and Molecularly diagnosed to know which fungi affected by solar sterilization,Study was made by covering the soil with a black mulch from june to august with (5)cm depth. Isolation after sterilization showed that fungi *Neoscytalidium dimidiatum*, Penicillium, Aspergillus, Rhizopus and Phoma, were affected with isolation (0)% from the covered soil, while the isolation from untreated soil was (80,5,10,5)% respectively for the same fungi., The maximum temperature recorded was (64.3) C for the covered soil compared to uncovered soil (42.6) C, while the lowest temperature recorded was (55) C for the covered soil compared to uncovered (39)°C, The isolated fungi were examined phenotypically by studying the nature of growth, colors, textures, and the edges of colonies, and the nature of the fungal hyphae. Then diagnosed based on international classification keys (Sutton and Dyko, 1989). when they matched the results of the molecular diagnosis. The analysis showed that diagnosed isolates belonged to: *Neoscytalidium dimidiatum*, *Alternaria alternata*, Curvularia, and *Fusarium. culmorum*. By comparing the sequences of nitrogenous bases with NCBI except Curvularia, were registered under the numbers (OR026671.1), (OR755780.1), and (OR828014.1). By calculating the percentage of dissimilarity and similarity between the fungi there was a genetic closeness between fungi, *Neoscytalidium dimidiatum* and *Fusarium culmorum*, at a rate of 0.12. In contrast, the fungi Alternaria alternate and Curvularia showed a percentage of genetic closeness of 0.06, while the percentage of divergence between the two groups was 0.20.

INTRODUCTION

Soil solarization is known as a physical sterilization method in which solar energy is used to sterilize the soil by heating it with solar rays, raising its temperature to a limit that kills pathogens (Katan, 2014; Tyagi and Raj,2021). It is one of the methods of sterilizing soil used in growing forest seedlings. It can be applied to greenhouses, and it is one of the cheapest methods of Sterilization (Dai *et al*., 2016) compared to chemical soil sterilization in which chemicals are used (Yim *et al*., 2017; Park *et al*., 2020; Daguerre, 2017). It is considered one of the most successful methods in the Arabic world due to the bright sun during the long summer and high temperatures. The method is summed up by covering the surface of the soil in the summer months with a transparent or black plastic polyethylene cover to retain the heat gained from the sun in the soil. The cover is tightly closed during the treatment

period and the soil is plowed well and softened with high moisture content. and the covering process continues for (4-8) weeks to obtain the desired results, as temperatures reach more than (50) C and a depth of (5-15) cm for the surface soil layer, and this is suitable for good pathogenic control, In this way, 40 fungal species, 25 species of parasitic nematodes, weeds, and a few pathogenic bacteria were controlled, as well as the fungus *Fusarium oxysporum* (Mahmoud *et al*., 2013; Bidima,2022; Baysal,2019). This process causes chemical, physical, and biological changes in the soil, and studies focus on biological changes because they are useful in eliminating moderately heat-tolerant Pathogenic fungi and stimulate beneficial fungi that can spread in the soil after the end of the sterilization period, such as Aspergillus, *Penicillium Glumus*, and Trichoderma. and maintain the biological balance in the soil, increase soil fertility, and exterminate pathogenic fungi that are moderately heat-tolerant (Strauss and Kluepfel, 2015), There are theories that explain what happens in the process of solarization of the soil. The first one depends on the rise in daytime temperatures to about 50 degrees Celsius and then their decrease to 30 degrees Celsius at night. As this process continues daily, something similar to the process of pasteurization occurs , As for the second theory, it depends on the gaseous emissions resulting from The activity of biological and chemical processes as a result of covering the soil with plastic that increases the concentration of gases on the surface of the soil, which leads to the killing of many pathogens (Yim *et al*., 2017), As for the molecular diagnosis of fungi, the technology of polymerase chain reaction (PCR) was used, which is one of the molecular techniques based on targeting and amplifying a specific region of the organism's genome to detect the various genetic relationships between the types of fungi, which can support the Phenotype diagnostic results. of fungi (Sebaih *et al*., 2016). This technique has been used to diagnose many fungi such as *Fusarium spp*. and *R. solani* and *Aspergillus spp*. (Al Hussaini *et al*., 2016). This technique used by (Al-Sawaf ,2022) to diagnose phytoplasma on tomato crops grown in the fields of the city of Mosul in northern Iraq, the match rate was 99.2%, and he was considered the first recording in Mosul of such a type.

MATERIALS AND METHODS

Materials and working methods

The agricultural media used in the study

The potato-Dextrose Agar (PDA) was the nutrient medium used from the Indian company Microxpress. It was prepared according to the manufacturer's instructions and stated on the special packaging. The medium was sterilized under optimal conditions with an Autoclave device.

Isolating fungi from soil

Isolation was carried out from random samples of soil, placed in plastic bags and then transported to the central laboratory of the College of Agriculture and Forestry at the University of Mosul. The indirect method was used, which is the Standard Plate Count (SPC) method. Its steps are summarized by adding 10 grams of soil to the first dilution bottle containing 90 ml of distilled water. and the bottle is shaken to obtain a homogeneous mixture. Then 10 ml of the first bottle is transferred using a sterile pipette and under sterile conditions to a second bottle containing 90 ml

of distilled water. It is shaken and in the same way, the dilutions are completed until the third dilution is reached for the purpose of growing it on the medium. PDA in Petri dishes with a diameter of 8.5 cm, Five drops of the solution were placed in the dish, then each sample was repeated three times. The dishes were incubated in an incubator at a temperature of 25±2°C. The dishes were examined after 5-7 days of incubation. The results were calculated by calculating the percentage of fungi isolated from the soil (Dar, 2009).

Molecular diagnosis using PCR technology for isolated fungi

Extracting DNA from fungi

Deoxyribonucleic acid was extracted in the central laboratory of the College of Agriculture and Forestry at the University of Mosul by using a kit produced by the Canadian company (Promega) and according to the company protocol. Standard PCR reactions were carried out starting with one thermal cycle, which is a preparatory cycle, and it was done at 96 degrees for 30 seconds for the purpose of initial denaturation of the DNA strand then was followed by 35 thermal cycles. Each cycle consists of three stages, The first stage was done at 96 °C for two minutes to denature the double strand, The second stage was done at 95°C for 30 seconds for the purpose of attaching the primers to its complementary site on the DNA strand. The final stage was at a temperature of 45°C for 30 seconds to begin the process of elongating the primer, and then comes a final thermal cycle at 72°C for 90 seconds for the purpose of completing the primer.

Primers design

Primers prepared by Promega company were used to prepare a polymerase chain reaction for the purpose of detecting fungi. The forward and reverse primers for the genes were designed based on the sequences of the nitrogenous bases. The complete coding sequence was obtained from the gene bank database at the National Center for Biotechnology Information.

components	Volume/µL	Concentration (ng/µL)
Extracted DNA	5	25-30
Forward primers	1	10 Pmol
Reverse primers	1	10Pmol
Deionized distilled water	23	--------

Steps for electrophoresis of DNA on agarose gel

The agarose gel was prepared after taking 1 gm of agarose powder and dissolving it in 100 ml of Tris boric acid EDTA buffer (TBE) solution. the templates were prepared for pouring the gel containing the comb at one end to make holes inside the gel layer, then the melted agarose poured and leaved it at room temperature. Then the rubber pieces were carefully removed after the agarose (gel) had hardened, and the mold containing the agarose (gel) was placed in the tank and immersed in the TAE solution. After making sure that the gel had hardened well, the comb was removed from the mold and the marker was prepared by adding 10 microliters of loading buffer, then it was injected into the side holes of the gel, and 12 microliters of the thermal cycling product samples were injected into the holes next to the marker. After that, the electrophoresis device was turned on after connecting its electrodes to

a 100 volt voltage source, and the DNA pieces were separated according to size. From the negative electrode towards the positive electrode, this takes approximately an hour and a half for the amplification results of the genetic material to appear. After the electrophoresis process is completed, the gel is carefully transferred to the UV Transilluminator, and pictures of the gel are taken with a digital camera.

Sequencing analysis

After obtaining the results, the nucleotide sequences of the five isolates were determined, and the packages resulting from the polymerase chain reaction were sent to the Canadian company Promega. The Sequencer Applicated Biosystem device was used, and the results were compared through the Internet with the database at the National Center for Biotechnology Information (NCBI), and matching was made to the Nucleotides sequence for the five isolates and identifying their types according to matching, and the fungus N was recorded. *N. dimidiatum, A. alternate,* and *F. culmorum* globally at the National Center for Biotechnology (NCBI) in the United States in the Gene Bank. In the name of the researchers and as attached in the appendix, the Phylogenetic evolutionary tree was then drawn based on the nucleotide sequences of the global isolates registered in the Gene Bank.

Fighting fungi using solar energy

The method of (Mahmoud *et al*., 2013) was adopted to sterilize the soil of forest nurseries using high bed nurseries with dimensions of (1 x 10) m at a rate of five treatments, including the cotrol, which was filled with soil and spread in the high cement beds with a height of 75 cm located in the forest department's nursery and flooded. With water up to the field capacity of the soil, it was prepared with a layer of black nylon, and the soil was covered with the sides fixed, for time periods of 30 days, 60 days, and 90 days, starting from June 1, to determine the efficiency of the sterilization time period. Isolation was done from the soil exposed to solar sterilization after the expiration of the sterilization period, separately and respectively, while the comparison treatment, the fungi were isolated from it for the same periods as before. Isolation was carried out at a depth of 5 cm from the surface soil after dividing the high beds with dirt dividers with dimensions of (1 x 1) m. The results were taken by calculating the percentage of isolation for all treatments and repeated at a rate of three replicates for each treatment. The experiment was carried out during the summer months for the period from 1/6 to 31/ 8.

RESULTS AND DISCUSSION

Isolation from the soil

Isolation was carried out randomly from the surface soil to a depth of 5 cm and after covering soil with black mulch, while the control treatment was left without covering. It is clear from Figure (1) the effect of solar sterilization after (90) days in combating soil fungi, where the percentage of isolation of the fungi *N.dimidiatum*, Penicillium, Aspergillus, Rhizopus, and Phoma, was (0)% from black coverage, while the percentages of fungal isolation in the comparison samples were (80, 5, 10, and 5)% for the fungi *N.dimidatum*, Penicillium, Aspergillus, and Rhizopus, respectively, despite the fact that the Phoma fungus was isolated from the black

coverage and At the end of July, with an isolation rate of (15)%, while the same fungi varied in isolation rates at the end of July.

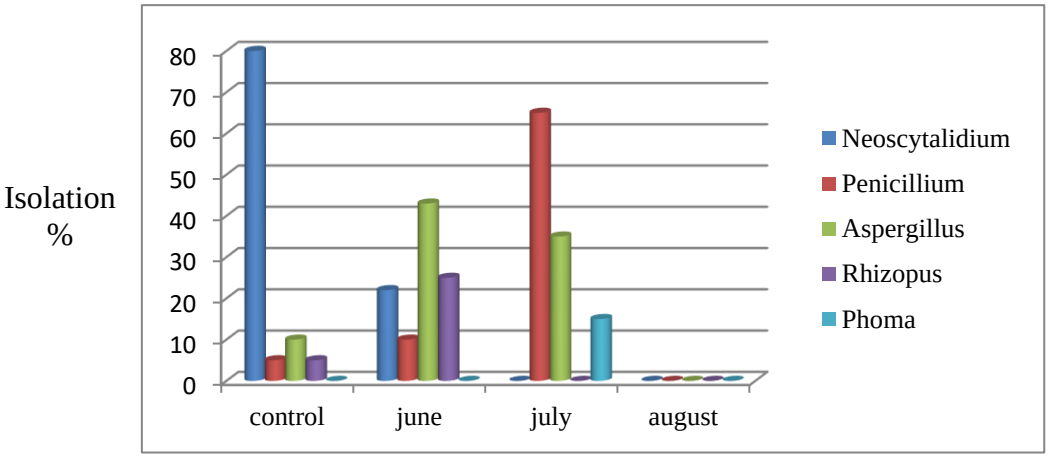

Figure (1): % of fungal isolation during the summer months (90 days) after black covering and comparison

We conclude from the above-mentioned figure that black coverage, after (90) days of coverage during the summer months, is one of the best methods of control through solar sterilization. Perhaps the reason for this may be due to the high temperatures within the coverage area because the black color absorbs the largest amount of solar rays. This is consistent with Mahmoud *et al*. (2013), where reproductive units decreased by (97.25%, 92.86%, and 94.43%) for the genres *Fusarium spp., Alternaria spp., Sclerotium spp.* respectively at a depth of 5 cm for solar sterilization of the soil.

Molecular diagnosis of isolated fungi

DNA extraction

After extracting the DNA of the isolated fungi in the central laboratory of the College of Agriculture and Forestry at the University of Mosul, the extraction was carried out using a kit produced by the Canadian company (Promega) and according to its protocol. they were transferred onto a 1% agarose gel and detected by exposing the gel to UV transilluminator. Five bands of DNA were obtained, and the results showed the possibility of amplifying the nucleic acid products (PCR product).

Polymerase chain reaction

After the DNA was extracted from the five isolated fungi, polymerase chain reaction electrophoresis was performed on a 1.5% agarose gel. To confirm the identity of the isolated fungi, the internal cloning region was amplified using primers ITS1 (TCCGTAGGTGAACCTGGG-3) and ITS4 (TCCTCCGCITATTGATATGC-3), and five Packages of DNA, as in Figure 2. The packages were 1500 base pairs in size.

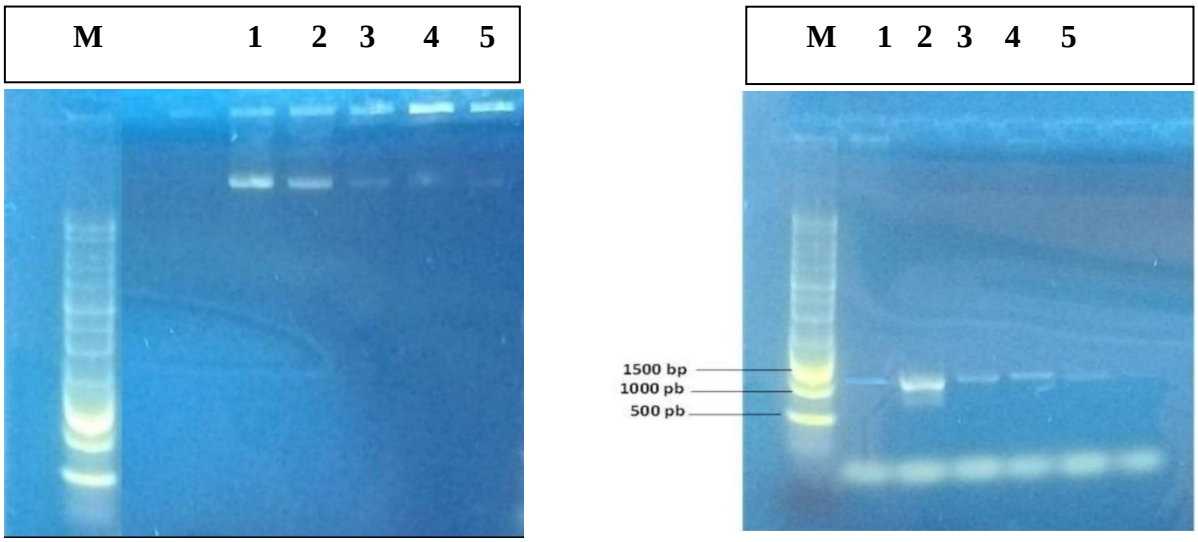

Figure (2): shows the results of polymerase chain reaction electrophoresis on a 1.5% agarose gel.
(M) represents the molecular weights imported from Promega, (1) F. culmorum, (2) Curvularia, (3) N. dimidiatum, (4) A. alternate, (5) Phoma.

PCR technology is one of the most important accurate diagnostic methods that uses universal primers, also called internal transcribed spacer (ITS), which is a barcoding sequence region and has been widely used as genetic marks in diagnosing Fungal species because this region is more suitable compared to the rest of the regions for diagnosing genera, and it is also preserved due to evolutionary constraints because it accurately distinguishes the genera and species related to them (Barbedo *et al*., 2016). It is clear from the diagnostic results that the molecular weights match by using a pair of general primers to detect genes in the internal transcribed space (ITS) region, as it showed a genetic match of the fungi that were isolated and diagnosed phenotypically with the sequence of the nitrogenous bases of the genes belonging to the fungi and targeted for confirmation. Genus and species for each of the following fungi. The first fungus, Fusarium culmorum, showed a genetic match to band 1300. The second fungus, Curvularia, showed a genetic match to band 1000. While the third fungus, N. dimidiatum, showed a genetic match to the band 1400. and the fourth fungus, Alternaria. alternata, showed a genetic match with the band 1400. The fifth fungus, Phoma, showed no genetic match after designing the primers. Table No. (1) shows a comparison of the genetic content of the fungal isolates, where the identified isolates belong to the following types of fungi:

Table (1): Molecular diagnosis of fungi based on the percentage of identity of nucleotide sequences with global isolates in the World Gene Bank

No.	Length	Sequence ID with compare	Identities	Source
1	549	ON844020.1	97%	N. dimidiatum
2	567	KR632488.1	100%	A.alternate
3	498	MG971283.1	100%	Curvularia
4	519	MK594970.1	99%	F. culmorum

Determine the sequence of nitrogenous bases

The sequences of the DNA samples of the isolated fungi were determined and compared with the sequences of the standard strains registered in the GenBank of the National Center for Biotechnology Information (NCBI) using the BLAST program. The PCR test showed that this isolate belongs to the fungus *N. dimidiatum* Figure (4-11), as there was closeness in the analysis process for these sequences with the sequences registered in the Gene Bank. The results of the analysis using the BLAST program showed a 97% match to the *N. dimidiatum* isolate registered in the Gene Bank. With the accession number (ON844020.1) as in Appendix (1), these sequences were registered in GenBank and the isolate was given the number (OR026671.1) as in Appendix (5). As for the isolate that belongs to the fungus *Alternaria alternata*, Appendix (2), There was a closeness in the analysis process for these sequences to the sequences in the Gene Bank, and the results of the analysis showed a 100% match, and they were registered with the accession number (KR632488.1), and they were registered in the Gene Bank, and the isolate was given the number (OR755780.1). As in Appendix (6). And the isolate belonging to the fungus Curvularia, as there was closeness in the analysis process for these sequences with the recorded sequences, and the results of the analysis showed a 100% match with the accession number (MG971283.1). As in Appendix (3). As for the isolate shown In Appendix (4), it belongs to the fungus *F. culmorum*, as there was closeness in the analysis process for these sequences with the recorded sequences. The results of the analysis showed a 99% match, with the accession number (MK594970.1) and registered in GenBank with the number (OR828014.1). As in Appendix (7), The results of the analysis showed that there were differences in some nitrogenous bases between the local isolates and similar standard strains in GenBank in some locations when comparing the sequences. This difference represents point mutations that occurred within the sequences of the nitrogenous bases of the local isolates and included replacing one nitrogenous base with another or deleting a base or adding a base, and these mutations occurred spontaneously (Al-Gader *et al*., 2018, Ami and guri, 2023). This technique was used by the researcher Alkhero, & Zainab. (2021) identified the following fungi*: Fusarium oxysporium, Fusarium solani, R. solani, N dimidiatum, Alternaria brassiciola, Trichoderma harzianum*. Al-Nema *et al* (2022) used PCR on the micro roots of cauliflower plants inoculated with agrobacterium rhizogenes for the purpose of obtaining high productivity. the following fungi were identified: *N. dimidiatum* with a match rate of 99%, the fungus *R. solani* with a match rate of 100%, and the fungus *Phoma exigua* with a match rate of 99% after isolating these fungi from the wood of eucalyptus, willow and sesame trees.

Percentage of genetic variation, similarity and difference

The evolutionary relationship of the evolutionary history of fungi was inferred by drawing the tree with certain drawing scales (Sneath and Sokal, 1973), where the lengths of the branches are in the same units as the evolutionary distances used, and the evolutionary distance was calculated using the complex probability method (Tamura and kumar, 2004), where the locations that are found are shown it has only one base for each minor branch next to each node in the tree. This analysis includes four sequences, and all ambiguous positions for each sequence pair have been removed (Tamura *et al*., 2021).

Abdulrazaq and Amen,2023 studied the genetic relationship between three types of birds chicken, guinea fowl, and quail using PCR technology, and found the highest percentage of genetic similarity was 0.608 between chicken and quail , also (Ibrahim *et al*, 2023) used the same technique to find the percentage of genetic closeness between two types of Ukrainian quail and the local brown quail , while (Abdullah *et al*, 2023), used the PCR technique to confirm the genetic transformation of Arugula plants after injecting the plant with agrobacterium rhizogenes bacteria, also (Hadi *et al* , 2023) used the PCR to study the effect of growth hormone gene on milk production and components by amplifying the gene based on the genome primer. Figure (3) shows the results of the genetic convergence of the two fungi, *N. dimidiatum* and *F. culmorum*, at a rate of approximately 0.12. The two fungi, Curviularia and *A. alternate*, also showed a genetic convergence of 0.06, while the rate of divergence between the two groups was 0.20.

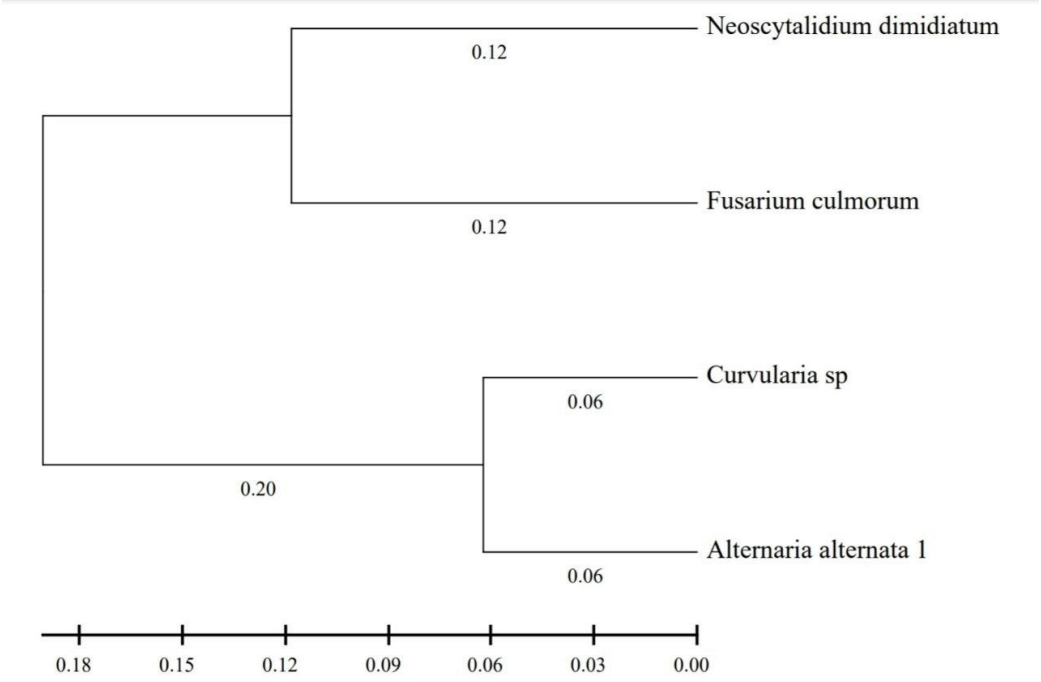

Figure (3): Analysis of the genetic kinship of the isolated fungi.

Temperatures measured during solar sterilization

It is clear from Figure (4) that temperatures increased during the covering process compared to the uncovered soil during the month of July. The maximum temperature recorded by the thermometer reached (64.3) C under the covering, while the comparison temperature during the same month was (42.6) C, and the lowest temperature recorded under cover. The coverage was (55)° C during the month of June, while the comparison treatment recorded the lowest temperature (39)° C. Also, the months of July and August recorded the highest temperature compared to the comparison treatment, and thus the black mulch is effective in raising the temperature to sterilize the soil, in addition to the time factor that played an effective role in killing pathogens in the soil, as well as the variation in temperature rates. Day and night, encourage the regrowth of dormant parts of the fungal structures that will be killed during the day due to high daytime temperatures.

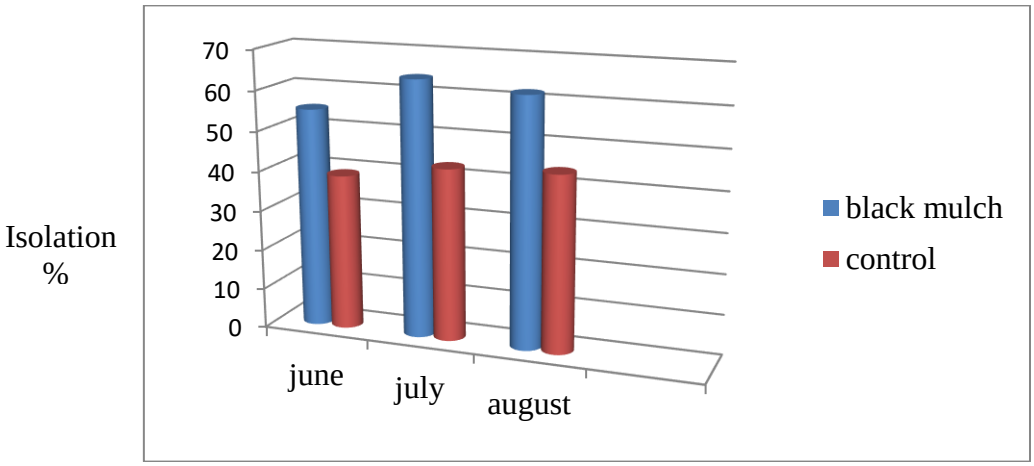

Figure (4): Comparison of temperatures during the months of solar sterilization

CONCLUSIONS

Black mulching after 90 days of mulching during the summer months is one of the best ways to combat fungi through solar sterilization, The months July and August in Mosul/Iraq are considered among the best months for the purpose of solar sterilization of the soil due to the high temperatures, Matching the phenotypic diagnosis of fungi with molecular diagnosis and keeping up with modern techniques in diagnosing fungi using PCR technology.

ACKNOWLEDGMENT

The authors sincerely thank the College of Agriculture and Forestry at the University of Mosul for providing laboratory facilities to accomplish this work.

CONFLICT OF INTEREST

We declare that we don't have any affiliation or entity with any organization regarding the financial or non-financial interest in the subject matter discussed in this article.

استخدام التعقيم الشمسي للتربة المعدة لشتلات الغابات وتأثيرها في تثبيط نمو فطريات التربة والتشخيص الجزيئي للفطريات المعزولة بتقنية ال PCR

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الخلاصة

استخدمت طريقة التعقيم الشمسي لغرض تعقيم التربة بوسائل جذرية قبل الزراعة للقضاء على المسببات المرضية في التربة و هدفت الدراسة الى عزل المسببات المرضية الفطرية المرافقة للترب غير المعقمة و تشخيصها جزيئيا و معرفة اكثر الانواع الفطرية تأثرا بالتعقيم الشمسي و اجريت العملية بتغطية التربة بطبقة من النايلون الاسود خلال اشهر الصيف وللفترة من 6/1 ولغاية 8/31 و لعمق 5 سم واظهرت نتائج العزل بعد

فترة التعقيم مكافحة الفطريات *Neoscytalidium dimidiatum*, Penicillium , Aspergillus , Rhizopus و Phoma وبنسبة (0) %عند التغطية وفي عينات المقارنة (80 و5 و10 و5)% وعلى التوالي و فقد تم مكافحة الفطر *Neoscytalidium dimidatum* و Rhizopus وبنسب عزل (0)%. وسجلت اقصى درجة حرارية خلال أشهر التعقيم و تحت التغطية خلال شهر تموز (64.3) مْ و درجة حرارة المقارنة خلال نفس الشهر (42.6) مْ و ادنى درجة حرارية سجلت تحت التغطية (55) مْ خلال شهر حزيران في حين معاملة المقارنة قد سجلت ادنى درجة حرارية (39) مْ ، وعند مطابقة الفطريات مع نتائج التشخيص الجزيئي فقد بينت نتائج التحليل ببرنامجBLAST ان العزلات المشخصة عائدة للفطريات التالية *Neoscytalidium dimidiatum* و *Alternaria alternate* و Curvularia و *Fusarium culmorum* وان العزلات سابقة الذكر بأستثناء Curvularia تم تسجيلها تحت الارقام (OR026671.1) و (OR755780.1) و (OR828014.1) على التوالي في قاعدة المركز الوطني لمعلومات التقنية الحيوية وتبين من وحساب نسبة التباين والتشابه بين الفطريات المشخصة وجود تقارب وراثي بين الفطرين *Neoscytalidium dimidiatum* و *Fusarium culmorum*بنسبة 0.12 في حين اظهر الفطرين *Alternaria alternate*و Curvularia نسبة تقارب وراثي بلغ 0.06 ، اما نسبة التباعد بين المجموعتين بلغت 0.20.

الكلمات المفتاحية: التغطية السوداء، تقنية تفاعل البلمرة المتسلسل، التعقيم الشمسي، فطريات التربة، تأثير درجات الحرارة.

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