Evaluating the efficiency of plants essential oils against Common fungal contamination affecting tissue culture of date palms (*Phoenix Dactylifera* L.) by *in vitro* culture

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Abstract
The study aimed to evaluate the in vitro activity of plants essential oils obtained from Thyme (Thymus vulgaris L.), Spearmint (Mentha piperita L.), Camphor (Cinnamomm camphora), Colocynth (Ctrullus colocynthis) and Rocket (Eruca vesicaria) against some important contamination fungus (Alternaria spp, Fusarium spp, Aspergillus spp and Penicilium spp) of tissue culture laboratory. Such plant essential oils were used at different concentrations (1, 1.5 and 2ml/100ml medium) and the percentage of mycelial growth inhibition of the fungus was tested. Results showed that all essential oils inhibited mycelial growth effectively. The mycelial growth of these fungi at concentrate 2ml/100 ml was fully inhibited by thyme and spearmint while camphor, colocynth and rocket were 89, 33.33 and 11.11 % respectively.

The results in in vitro showed that thyme, spearmint and camphor prevent contamination from occurring absolutely, while the browning coloration appearance on the plantlets which were 100, 100 and 96,67% respectively were compared with the control (without essential oil) which was 3,33%. The growth characteristics of the plantlets (number of shoot branches and shoot length) were also not observed in these treatments. At the last stage of the experiment, plantlets (with essential oils) withered and led to death.

Keywords: Essential Oils, Contamination Fungal, Tissue Culture, Date Palms.

Introduction

The use of plant tissue culture technology contributes significantly to the growth, multiplication and maintenance of plants. Date palm plants for commercial and other purposes *in vitro* with this technology can produce a large number of high-quality plants within a short period.[29] Tissue culture media, which contain a high concentration of sucrose, support many micro-organisms (such as bacteria and fungi) in growth. Usually these microbes grow faster than the cultivated tissue when the media enters and eventually kills them. The pollutants release metabolic waste which is harmful to the tissues of plants. Therefore, preserving a fully aseptic environment through the techniques of cell culture is absolutely essential. The medium includes many possible sources of contamination, the culture vessel, medium itself, the explant, the transfer area environment, the tools used in the handling of plant material during inoculation, subculture and the culture room environment.

While various sterilization techniques such as chemical, moist heat sterilization or filter sterilization are used at different micropropagation stages to ensure clean cultures, the contaminants may be introduced by microarthrobod vectors or endophytic microbes in the cultures with explants during laboratory manipulation.[26,36,40] The key microbial contaminants frequently reported in the plant *in vitro* cultures are bacteria and fungi.[9,31,32]

Major bacterial contaminants are *Pseudomonas syringae*, *Bacillus licheniformis*, *B. subtilis* and *Erwinia sp*. whereas the fungi are *Alterneria tenius*, *Aspergillus niger*, *A. fumigatus* and *Fusarium culmorum* are frequently observed in plant tissue culture by Odutayo et al.[30]

Essential oils have been shown to exert biological activity against plant fungal pathogens in *vitro* and in *vivo* and can be used as bio-fungicides.[16,22,34] Such products are generally considered more suitable and less harmful to the environment and could be used as alternatives to treatment of plant diseases.[11]

Wilson et al[42] reported that essential oil extracted from red thyme (*Thymus zygis* L.) has a major inhibitory effect on germination of *Botrytis cinerea* spores compared with other essential oils. Studies conducted by Abdelgaleil[2] and Soad and Abdelgaleil[38] verified that antifungal efficiency of eight essential oils was tested against ten phytopathogenic fungi, the concentration of 500 ppm of *Mentha microphylla* essential oil suppresses the mycelial growth of *F. culmorum*, *F. oxysporum*, *Penicillium digitatum*, *R. solani* and *Rhizopus stolonifer*.

Katooli et al[24] showed that eucalyptus essential oil in all tested had totally inhibition effect on mycelial growth of *Pythium ultimum* and *R. solani*. Barrera-Necha et al[8] studied the antifungal effects of ten essential oils against *F. oxysporum* and *F. gladioli*, they found that essential oils of cinnamon, clove and thyme were inhibited the mycelial growth of *Fusarium* sp. totally. Abdel-Kader et al[3] also verified that thyme and spearmint essential oils inhibited the

mycelial growth of *F. solani*, *R. solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina* under *in vitro* culture conditions.

Jagana et al[21] investigated five essential oils in *vitro* as well as in *vivo* against *Colletotrichum musae*, they found complete inhibition of mycelial growth; they also found that essential oils of clove at all the concentrations tested (0.5, 1.0 and 2.0 %) and eucalyptus at 2.0% concentration caused complete inhibition of mycelial growth of this contaminant.

The present study has been conducted at the laboratories of Date palm research center, Basrah University to investigate and identify various sources of microbial contamination prevalent and control of the effectiveness of some plant essential oils on these contaminations. It also envisages reducing the monetary losses incurred by pollution in the plant tissue culture laboratory.

Material and Methods

Purification of Fungal Contaminants: The fungal contaminants were detected by the highest frequency of occurrence when incubated into Potato Dextrose Agar (PDA). The isolates were purified by a series of transfers to the fresh culture medium. The identification of fungal contaminants was done 4-7 days after transferring into the fresh medium when pure cultures were obtained.[7,15] Isolation frequency (IF) for each fungus was determined and expressed as percent by using the following formula:

IF = Number of samples occurrence of fungi species / Total number of samples *100.

Essential oils: The pure essential oils were spearmint, thyme, colocynth, rocket and camphor oil obtained from the local market. These essential oils were exposed to UV radiation for 10 min and then used for the experiments. These oils were selected based on the literature survey.

Antifungal activity of essential oils: PDA medium with 1, 1.5 and 2 ml/100ml concentrations of the essential oils such as thymus, spearmint, colocynth, rocket and camphor oil was prepared. Tween-20 (Sigma) was incorporated into the agar medium to enhance oil solubility and allowed to solidify. About 20 mL of the medium was poured into each Petri dish. Five mm disks of 5-day culture of the test fungi from the edge of the plates were placed in the center of the Petri dishes and incubated at 28^{o}C for growth. The colony diameter was measured in mm after incubation. For each treatment, three replicates were maintained. PDA medium without the essential oil served as control. The observations were recorded every 12 hours after complete growth of control treatments. The rates of mycelial growth inhibition (GI%) were calculated by the following formula:[20]

Growth inhibition %= dc−dt/dc *100

where dc = Average increase in mycelial growth in control and dt = Average increase in mycelial growth in treatment.[37]

***In vitro* evaluation of essential oils:** After sterilization treatments, the nodal were cultured on MS basal medium supplemented with sucrose 40 g /L, adenine sulfate 40, myo-inositol 100, $NaH_2PO_4.2H_2O$ 170, activated charcoal 500mg/L, agar-agar 7 g /L , vitamins 10 ml /L, benzene adenine 0.5 mg /L, kinitine 0.5 mg /L and naphthalene acetic acid 1.5 mg /L.[28] pH of media was adjusted to 5.8 with NaOH prior to autoclaving. The described media were poured into culture glass bottles (approximately 50ml per bottle), 2 ml concentration of each essential oils (thyme oil, spearmint oil and camphor oil) was added to each bottle. Tween-20(Sigma) was incorporated into the bottles to enhance oil solubility. Control treatment were without any disinfecting agent, all bottles closed with screw caps.

Then, the media (bottles) were autoclaved at 121^{o}C and 1.05 kg.cm^2 for 20 minutes. After cooling and solidification, tissue cultural plantlets were planted directly in these bottles. Each treatment had four replication with three plantlets in each replicate. All bottles were placed under appropriate conditions in the growth room, the following characterization was recorded at the end of experiment:

1 - % Contamination, 2- % Brown coloration, 3- Number of shoots and 4-Shoots length.

Experimental design and Statistical analysis: All experiments were repeated three times; experimental design was completely randomized design (CRD) using SPSS software. Significant differences between transformed values were detected and significance was set at 5% probability.

Results and Discussion

Fungal contaminant isolated from tissue culture: Table 1 details the cultural and morphological characteristics of the fungal isolates. The results agree with survey of fungal contamination of various date palm cultivars during embryogenic callus production, showing that the most prevalent species fungi were *Aspergillus niger, A. clavatus* and *Alternaria alternata* as contaminants of six separate date palm cultivars including Um Al-Dihin, Shwaythee, Breem, Barhi, Hilawi and Al-Sayer.[18]

Abass et al[1] research showed that the *Aspergillius niger, Penicillium sp.* and *Alternaria alternata* were the pollutants with concentrations of 27%, 25% and 18% respectively whereas *Aspergillius terreus* was at the lowest level. A recent study with Al-Mayahi et al[5] identified various genera of fungi from polluted date palm tissue culture such as *Aspergillius niger*, *Chaetomium atrobrunneum*, *Penicillium* sp. and *Fusarium* spp.

Antifungal activity of Essential oils: The activity of the tested essential oils against the three fungus contamination is summarized in table 2. The inhibitory effects of plant essential oils were tested at different concentrations. Essential oils from thyme, spearmint and camphor plants

showed a high activity against *Alternaria* spp ,*Fusarium* spp and *Aspergillus* spp at the concentration 2ml/100ml and did not show any mycelial growth .

The results of the other essential oils showed a minimum inhibitory concentration(MIC) at all tested concentration. The IMC value were lowest at colocynth and rocket. Our results agree with Azizi et al[5] who verified that radial growth of *P. italicum* was totally inhibited by thyme (500 mg/L), *Saturega hortensis* and *Thrachryspermum copticum* (1000 mg/L). Moreover, Abdolahi et al[4] showed that the thyme essential oils had good antifungal activity against *Botrytis cinerea* and *Mucor piriformis*.

According to Šegvić et al[35], the main components of essential thyme oil containing p-cymene (36.5%), thymol (33.0%) and 1,8-cineole (11.3%) and pure thymol were antifungal activity, Pure thymol displayed an inhibition roughly three times stronger than basic thyme oil. The results of Kuinke et al[25], showed that the plants essential oils harbored the mycelial growth inhibiting fungal toxic theory, the toxic properties of the *thymus linearis* and *mentha arvensis* were found to be more effective, the extract of *T.linearis* and *M.arvensis* totally inhibited the mycelia growth of the fungus *Glomerella cingulate*.

Study of Mahilrajan et al[27] showed Camphor oil to be the most powerful essential oil on the *A. niger, A. flavus* and *Peniillium* spp an average growth inhibition of 100, 96.38 and 84.99 % respectively.

***In vitro* evaluation of essential oils:** The results in table 3 showed that the three plants essential oils caused 100% growth inhibition on all species of fungi at 2ml/100ml while control treatment recorded 10% of fungal contamination. Our research coincides with findings from Taghizadeh and Solgi[39] who used thymol and carvacroal for disinfection of *Cynodon dactylon*. Deein et al[13] examined effect of essential oils as disinfecting materials on sterilization of MS medium and growth of *chrysantimum internodes*. On the other hand, all these treatments significantly exceeded the control treatment in the percentage of browning coloration which were 100, 100 and 96.67% respectively compared with control which was 3.33%.

The results (table 4, fig. 1) showed total inhibition of growth and development of plantlets in all plants essential oils treatments since the control reported a substantial superiority in the average number of shoots branches and shoots length were registered in all essential oils treatments. The interpretation of likelihood of this finding agrees with the explanation of many studies showing that the allochemicals are mostly plant secondary metabolites (PSM) of either acetate or shikimate metabolic pathways.

Such chemicals usually involve long-chain fatty acids, phenolic compounds, alkaloids, hormones and coumarin derivatives, quinines, flavonoids, tannins, terpenes and water-soluble organic acids with a wide range of action.[11]

Table 1
Frequency of fungi isolated from contamination samples of tissue culture medium

Associated fungi %			
***Alternaria* sp**	***Fusarium* sp**	***Aspergillus* sp**	***Penicillium* sp**
45	32	17	6

Table 2
The effect of essential oils against the contamination fungal on PDA medium.

Essential oils	Conce. ml/100ml	Percentage of inhibition Species			Mean of Inhib. perc.
		Alternaria	***Aspergillus***	***Fussarium***	
Thymus	1	66.6	66.6	100	77.7
	1.5	77.7	88.8	100	88.8
	2	100	100	100	100
Spearmint	1	66.6	100	77.7	81.9
	1.5	77.7	100	88.8	88.8
	2	88.8	100	100	96.2
Colocynth	1	33.3	22.2	22.2	25.9
	1.5	55.5	22.2	33.3	37
	2	55.5	33.3	33.3	40.7
Rocket	1	33.3	22.2	11.1	22.2
	1.5	22.2	11.1	11.1	14.8
	2	22.2	11.1	11.1	14.8
Camphor	1	66.6	66.6	66.6	66.6
	1.5	88.8	77.7	77.7	81.4
	2	89	88.8	89	88.9

These PSMs can be found or extracted from every part of the plant including roots, rhizomes, stems, leaves, flowers and seeds.[10,41] Allelochemicals may interfere with various important processes such as plants growth, photosynthesis, respiration, water relations, ion uptake and growth, cell ultrastructure and oxidative stress.[33,43]

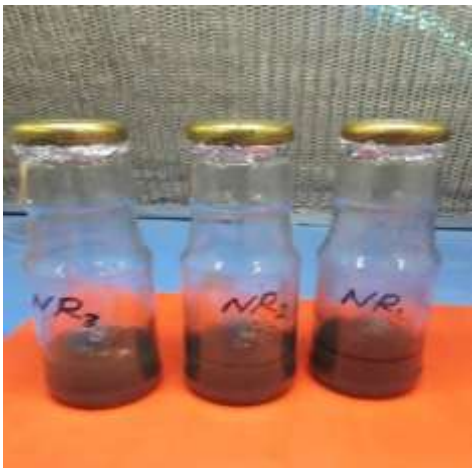

Fig. 1: The effect of plants essential oils treatments on tissue culture plantlets.

That can also be attributed to the presence of plant essential oils in chemical compounds. Thyme produces a lot of phenol compound and can make them more stressful. The products from this oxidation interrupt the function of the enzyme and cause tissue necrosis, loss of explant establishment in medium and eventually explant death.[19]

Our results are in line with Karaca et al[23] who found that highest doses (5-10%) of clove, mint and oregano oils inhibited completely fungal growth in wheat seeds, also inhibited seed germination. Other research by El-Bakry et al[14] found that all essential oils tested had side effects on the germination and growth of wheat seedlings.

Conclusion

The results obtained from both experiments indicated that spearmint, thyme and camphor essential oils inhibited the mycelial growth and fungus contamination of tissue culture; also the inhibition of mycilial growth increased significantly with the increase of essential oils concentrations. While there is an important problem happening in tissue culture experiment at the latter stages of the experiment, all the plantlets which are treated with plants essential oils are wilting gradually and at the end leading to death.

Table 3
Effect of plant essential oils on percentage of contamination and brown coloration.

Treatments	Contamination%	Brow coloration%
Spearmint oil	0.00	100
Thyme oil	0.00	100
Camphor oil	0.00	96.67
Control (without oil)	10	3.33
LSD	0.941	3.843

Table 4
Effect of plant essential oils treatments on shoots number and shoots length in *vitro* culture technique.

Treatments	Shoots number	Shoot length
Spearmint oil	0.00	0.00
Thyme oil	0.00	0.00
Camphor oil	0.00	0.00
Control(without oil)	5.67	3.57
LSD	1.087	0.661

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