In vitro, effect of sucrose concentration and type of culture medium on fungal colonies infections on Murashige and Skoog medium

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Abstract

This study was conducted in the laboratories of the College of Agriculture and Marshlands, University of Thi Qar, South of Iraq. The paper presents the effect of sucrose concentration and culture medium on the colonies of fungal infections in MS medium (Murashige and Skoog, 1962) basal medium supplemented with four variants of sucrose (0 control, 10, 20 and 30) gm/l as carbon source were tested. Two types of MS(1. solid 7 g/l of agar was added 2. liquid) were used, without any hormones and vitamins. For the purpose of fungal growth, the culture media were exposed to laboratory air for two hours, then the samples were incubated at a temperature of 27 °C for 7 days. Results shown there are 5 genuses (Aspergillus spp, Rhizopus spp, Fusarium spp, Mucor spp and Penicillium spp) that grow mainly on MS culture medium. Also, the results confirmed the existence of a significant correlation between fungal growth and sucrose concentrations, the comparison treatment 0 g/L (control) did not record any fungal growth, while (30 g/l) concentration was recorded the highest growth rate (liquid:31.30 mm and solid:19.60 mm).

Keywords: *genus, culture medium, MS. Aspergillus, Rhizopus, Fusarium, Mucor, Penicillium.*

1. INTRODUCTION

Micropropagation is a technical process for producing large quantities of plants quickly and similar to the mother plant and free of diseases through the cultivation of cells, tissues, organs, embryos or seeds. (Abdalla et al., 2022; Pe et al., 2020; Rani et al., 2019). This technique is widely used in the production of plants that do not produce seeds or are difficult to propagate by means of normal sexual or asexual reproduction (Dhiman et al., 2020; Bridgen et al., 2018; Bidabadi and Jain, 2020). There are many cultures media are used in the technique of tissue culture that differ based on their organic and inorganic components and the purpose of cultivation (Al ghasheem et al., 2022) such as: White Medium (White, 1943); MS medium (Murashige and Skoog, 1862); LS medium (Linsmaier and Skoog, 1965); B5 medium (Gamborg et al., 1968) and NN medium (Nitsch and Nitsch, 1969). MS medium was discovered by Murashige and Skoog in 1962. It is widely used in the tissue culture laboratories. MS media is content a blend of nutrients such as inorganic salts, carbohydrates, amino acids and vitamins. MS

culture media is used to induce organogenesis; callus culture; micropropagation and cell suspension (Kyzioł et al., 2021; Fortini et al., 2021; Bidabadi and Jain, 2020). Contamination is one of the biggest challenges facing the micropropagation technique (Marshall et al., 2021; Seliem et al., 2020; Al ghasheem et al., 2018). Contamination arises in the micropropagation technique from several sources, such as explants; tools and workers in the laboratory, culture media and laboratory air (Loyola-Vargas and Ochoa-Alejo, 2018; Venat et al., 2018). Microorganisms such as viruses; viroids; prokaryotes (bacterial and bacterial agents) and fungi infect the cultures tubes, causing the failure and death of explants used. Contamination is a problem that causes loss of time and increases the cost of production (FAO/IAEA, 2022; Gangopadhyay et al., 2017). These microbes are the most common tissue culture problem due to their small size and ability to grow rapidly. The weakness of explants due to the cutting of the tissues in them and the presence of nutrients ready for absorption in the culture media help in creating an environment suitable for the growth and opportunistic invasion of these microorganisms (Babao et al., 2001). Fungi are environmental microorganisms that play an essential role in nature's ecosystem. They deplete and damage plant and animal tissues, produce mycotoxins, and possibly desired biological transformations (Tanprasert and Reed, 1997; Leifert and Cassells, 2001). Therefore, it is important to know their nutritional needs. Fungi in general consume carbohydrates on the form of sugars (Hashimoto et al., 2005).

Carbohydrates are important components in the growth and development of fungal pathogens because these sugar compounds are the metabolites that are used as carbon skeletons for biosynthesis and formation of other compounds, provide substrates for energy production, and enhance cellular pathways in fungi (Goulet and Saville, 2017). Studies have confirmed that high levels of sugars affect the secretion of enzymes by fungi that degrade the host's cell walls (Kikot et al., 2009; Carapito et al., 2009). Researches confirm that: I. The effect of soluble external sugars on the growth and formation of spores (formation of macroconidia and microconidia) of fungi and it has an important role in increasing or decreasing the growth, development and spread of fungi; II. High levels of sucrose stimulate plant defenses against fungal invasion and attack of cells and tissues. Sugars act as metabolic signaling molecules in plant cells that stimulate gene expression, including defense genes (Ehness et al., 1997; Biemelt and Sonnewald, 2006; Johnson and Ryan, 1990; Morkunas and Ratajczak, 2014); III. Through the role of sugars in supplying Plant energy through the process of respiration (Morkunas and Bednarski, 2008; Morkunas et al., 2008; Morkunas et al., 2013; Swarbrick et al., 2006); IV. Polysaccharides also provide the carbon skeleton for building defense compounds such as flavonoids, lignin and stilbene (Jeandet et al., 2018; Jeandet et al., 2020; Morkunas et al., 2005; Morkunas et al., 2007;Morkunas and Gmerek, 2007; Morkunas et al., 2011). Aims of study to isolate and identify fungi that attack the MS culture medium in order to choose the appropriate pesticide for it and tested role of sucrose and type of MS culture medium (solid-liquid) on the growth of these fungi.

2. Materials and Methods

2.1. Culture media and culture conditions

The experiment was conducted in the laboratories of the College of Agriculture and Marshlands, University of Thi-Qar, South of Iraq. MS cultures media (Murashige and Skoog, 1962) basal medium supplemented with

four variants of sucrose (0 control, 10, 20 and 30) g/l as carbon source were tested. Two types of MS (1. solid 7 g/l of agar was added 2. liquid) were used, without any hormones and vitamins (Tables. 1 and 2). Also, antibiotic Amoxicillin (250mg/l) were added to the culture medium to inhibit the growth of bacteria. For the purpose of knowing the genus and species of fungi that infect the tissue culture media, MS culture medium was poured into Petri dishes. Petri dishes were exposed to laboratory air for two hours to the purpose of allow fungi that have the ability to grow in a culture medium whose pH is 5.7, then incubated at a temperature of (2 ± 25) C for 7 days. After 1 week of incubation, fungal colonies growing in MS culture medium containing different concentrations of sucrose were identified on the basis of their cultural and phenotypic characteristics according to different identification keys (Booth 1966; Dhingra and Sinclair 1978; Nelson et al. 1983; Ellis, 1971,1976; Domsch et al., 1980; Agrios 2004; Nasrawi 2006). To obtain pure fungal growths, the serial spore dilution method was used, whereby 1 ml of the diluted growth was mixed sequentially with dissolved Nutrient agar and transferred to sterilized plates (Choi et al. 1999).

For the purpose of classifying and diagnosing the isolated fungal species, part of the colony isolates were transferred and placed on glass slides containing Lactophenol cotton blue with violet crystal stain, then examination using light microscopy. Record the effect of sucrose on the growth and activity of fungal colonies using the colony diameter measurement technique (Trinci, 1969).

Table 1. Scheme of Variants for concentration of sucrose added to MS (solid and liquid) culture medium

Variants	Components of MS
V1	MS+7 agar+0.0 g/l sucrose
V2	MS+7 agar+10.0 g/l sucrose
V3	MS+7 agar+20.0 g/l sucrose
V4	MS+7 agar+30.0 g/l sucrose
V5	MS+0.0 g/l sucrose
V6	MS+10.0 g/l sucrose
V7	MS+20.0 g/l sucrose
V8	MS+30.0 /l sucrose

Table 2. Mineral chemical components of MS culture medium

Macronutrients	Components (mg/l)
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
$MgSO_4.7H_2O$	370.0
KH_2PO_4	170.0
Micronutrients	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8

2.2. Statistical analysis

The study was conducted using a complete randomized design (CRD), the results of the experiment were analyzed statistically by the percentage of developing fungi was measured with estimating the average diameters of the fungal growths using the statistical analysis program SPSS version 14, and the experiment was repeated three times. The two-way ANOVA test was used, Using the least significant difference test between the means (LSD)at the probability level of 0.05 (Al-Rawi and Khalaf, 2000).

3. Results and Discussion:

3.1. Isolation and identification of fungal growths

The results showed that (Table. 3) there are 5 genuses (Aspergillus spp, Rhizopus spp, Fusarium spp, Mucor spp and Penicillium spp) that grow mainly on MS culture medium. Where the genus (Aspergillus spp 30%) recorded the highest growth rate of fungal colonies, while the genus (Penicillium spp 10%) recorded the lowest value of fungal growth, because airborne Aspergillus conidia are protected from UV radiation because their cell walls contain melanin (Bhabhra and Askew, 2005). Studies show that the melanin layer, known as the "fungal shield" that is produced by oxidation of the amino acid tyrosine, followed by polymerization is due to the ability of the polymer to provide protection from several stressors to which fungi are exposed including low temperatures and UV radiation (Robinson, 2001).

3.1.1. Aspergillus

The results of the study showed that (A. niger and A. flavus) constituted 84% over the rest of the species of the genus Aspergillus. The results of the study are consistent with what was found by (Nitsche et al., 2012; Gniadek et al., 2017). A. niger is a filamentous fungus that grows in the form of black aggregates or colonies that produce organic compounds and enzymes that are harmful to living tissues, can to grow and survive in different environments as temperature range12–65°C and pH 2.1 - 8.8 (Schuster et al., 2002; Frisvad et al., 2002; Kwon-Chung and Sugui, 2013). Aspergillus is a filamentous fungus that spreads widely in the soil, seeds, grains and decaying plants, where it grows as saprophytes. They mainly attack plants, but there are species that infect humans and pose a threat to their lives, such as A. fumigatus (Pitt, 1994; Heitman, 2011; Seyedmousavi et al., 2015). Many species of Aspergillus spp is live long periods of time in the soil without any host (Wicklow et al., 1993), but when the right conditions are present, the sclerotia germinate to form mycelia that then form conidiophores, which spread through the air through the wind to infect plants and seeds (Amaike and Keller, 2011).

3.1.2. Rhizopus spp

The results confirmed that (17%) of the isolated fungal colonies were of the genus (Rhizopus spp). The results showed that, the genus Rhizopus is characterised by the presence of stolon and pigmented rhizoids, the formation of sporangiophores, singly or in groups from nodes directly above the rhizoids. Colonies of Rhizopus spp were appeared in fast-growing and dense, cottony with a white to gray or brown color, depending on the species. There were colonies growing in concentric rings containing brown umbrella-like spores and conidia. The results of the study are consistent with what was found by (Odebode et al., 2020). The reason for the growth of fungal colonies on MS culture medium containing sucrose is due filamentous fungi can take advantage of carbon sources available in the nature (Fang et al., 1998).

3.1.3. Fusarium spp

The results confirmed that (15%) of the isolated fungal colonies were of the genus (Fusarium spp). These isolates were confirmed that the species (F. solani, F. chlamydosporum and F. oxysporum) are the most common species growing on MS culture media. Fungal Colonies were characterized by a creamy white color with red, green and purple pigmentation, depending on the species. These results are similar of studies (Trabelsi et al., 2017). Fusarium spp belong to ascomycetes. There are

more than 70 species belonging to this genus, And a few of them cause diseases to plants, humans and animals (Zhang et al., 2006; Nucci et al., 2015; Munkvold, 2017). Fusarium spp has the ability to produce mycotoxins in high quantities that cause virulence factors to the infected explants (Hof, 2008; Munkvold, 2017).

3.1.4. Mucor spp

The results confirmed that (15%) of the isolated fungal colonies were of the genus (Mucor spp). The results of the study showed that the fungal colonies of Mucor spp were white or gray in color, fast growing, consisting of thin threads carrying spores. Due to the growth and development of the spores, the old colonies become gray to brown. Mucor spp were contains combinations of formation of non-apophysate sporangia on simple or branched sporangiophores by zygospores that were borne on opposed or tong-like suspensors. The results of this study were similar to studies (Schipper, 1978; Walther et al., 2013).

3.1.5. Penicillium spp

The results confirmed that (10%) of the isolated fungal colonies were of the genus (Penicillium spp). The results confirmed that (Penicillium spp) grows in a branched filamentous form containing round, single-celled conidiospores. Penicillium reproduces asexually. The results of this study were similar to those of Khashba et al. (2018) found that Aspergillus spp was the first genus (100%) in Egypt, and Penicillium spp was the second genus, which had a frequency of 92.5%.

Table 3. Percentage of invasive fungal genuses in MS culture medium

Genus	Family	% of fungal growth
Aspergillus spp	Trichocomaceae	30%
Rhizopus spp	Mucoraceae	17%
Fusarium spp	Hypocreaceae	15%
Mucor spp	Mucoraceae	15%
Penicillium spp	Trichomaceae	10%
others	-	13%
Total	-	100%

3.2. Effect of sucrose on fungal growth

The results of this is study showed that there were significant differences between the concentration of sucrose and the growth of fungal colonies, as the study showed that all sugar concentrations had a deterrent effect on the growth of fungal colonies and the relationship was positive. 30 gm/l sucrose was recorded the highest value of fungal growth compared to the rest of the concentrations: V8 (liquid) 31.30 mm and V4 (solid)19.60 mm), while no fungal growth was observed in the comparison samples 0gm/l(control). The study also concluded that the sucrose-free medium did not record any fungal growth despite the presence of spores and reproductive parts in the same medium (Fig. D), and this indicates that the fungal growth (grows and develops) on the availability of sugars wherever they are found, despite the availability of other mineral elements. These results can be applied in tissue culture technique to reduce contamination by fungi through the use of sucrose-free culture media in the initiation stage. Studies (Gupta and Neha, 2012) indicated that polysaccharides increase the growth of colonies and the entire biomass and decrease the level of polysaccharides in the surrounding environment (Table. 4).

Also, the results of the study confirmed that the fast growing fungal growths in MS medium that contains high concentrations of sucrose prevent the growth of other fungi near it (Fig. A), The results of the study also confirmed that the low concentrations of sucrose in the culture medium increased the number of fungal colonies compared to the culture media that

contained high concentrations of sucrose:V2 (solid)=21.36 and V6(liquid)=15.67 colonies /dush (Table. 5 and Fig. B). The results of this study are similar to the studies of Mehrotra and Kumar (1961), when they found the effect of sugars on increasing the growth and biomass of fungi. Studies confirm that during the growth of microorganisms produce substances that are either inhibitory or stimulating for themselves or for other organisms (Gwynne-Vaughan, 1922; Clark, 1900). There are a lot of interactions that can be adopted when a fungus grows in close proximity to other fungi. Growth is usually investigated when more than one type of fungus is adjacent. One may inhibit the other or the growth of one may be impeded on the growth of the other. Pommerrenig et al (2020) indicated that soluble sugars are a source of carbon for pathogens, which increases their production of enzymes and thus their virulence fungal colonies are physiologically diverse microorganisms that can grow as long, branched, thin growths of fungal mycelium (Schrickx et al., 1993).

3.3. Effect of culture medium type on fungal growth

The results of the current study indicated that there is a statistical significance between the growth of fungal colonies and the type of culture medium, where the liquid medium was significantly superior to the solid medium in the growth rate of fungal colonies: liquid=17.40mm and solid=10.70mm (Table. 4), the reason for this may be due to the fact that the mineral nutrients and sucrose in the liquid culture medium are free to move, which makes the fungal mycelium move and develop faster. This is results was similar to the studies (VanderMolen et al. 2013) when they used solid and liquid media on the growth of fungal isolates. Also, previous studies have confirmed that the type of culture medium has primary effects on the induction of secondary metabolites in fungi (Bode et al., 2002; Miao et al., 2006).

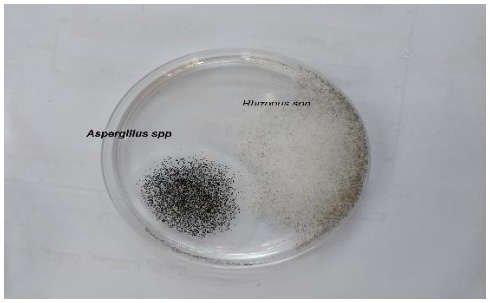
Table 4. Effect of sucrose concentration and type of MS culture medium on the growth diameter of fungal colonies (mm) after 1 week from incubation

MS Type of	Sucrose mg/l				Mean
	0.00	**10.00**	**20.00**	**30.00**	
Solid	0.00	09.80	13.60	**19.60**	**10.70**
Liquid	0.00	14.20	24.10	**31.30**	**17.40**
Mean	0.00	12.00	18.80	25.40	
LSD	MS	9.060	Sucrose	5.716	

Table 5. Effect of sucrose concentration and type of MS culture medium on number of fungal colonies after 1 week from incubation

MS Type of	Sucrose mg/l				Mean
	0.00	10.00	20.00	30.00	
Solid	0.00	**15.67**	11.30	8.23	**8.80**
Liquid	0.00	**21.36**	9.42	3.55	**8.58**
Mean	0.00	18.51	10.36	5.89	
LSD	MS	6.550	Sucrose	2.662	

Figures: A. V8 (MS+30.0 g/l sucrose) Competition between (Aspergillus spp and Rhizopus spp) by enzymes produced by fungal growths. B. V2 (MS+7 agar+10.0 g/l sucrose), Aspergillus spp grows in solid culture media with low concentrations of sucrose, it is noted that the fungal colonies are small in size with an increase in their number. C. Measurement of diameters of fungal colonies growths. D. V5 (MS+0.0 g /l sucrose) No fungal colony growth.



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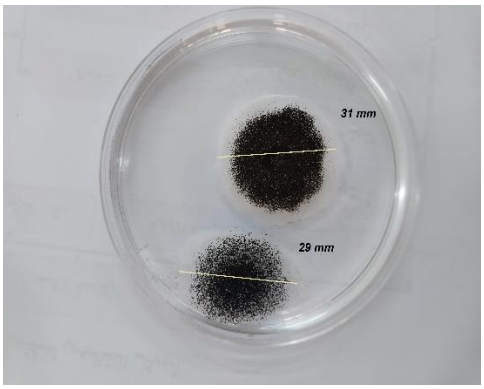

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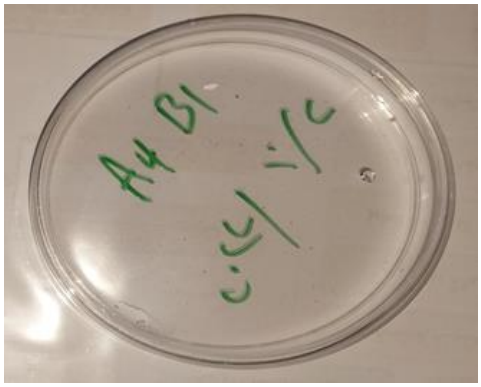

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4. Conclusions

Results shown there are 5 genuses (Aspergillus spp, Rhizopus spp, Fusarium spp, Mucor spp and Penicillium spp) that grow mainly on MS culture medium. From these results, we can conduct new tests to obtain a combination of fungicides and use them for surface sterilization of explants and add them with the culture medium to prevent contamination caused by this genus. Also, the results confirmed the existence of a significant correlation between fungal growth and sucrose concentrations, as the comparison treatment excelled by not seeing any fungal growth, while the concentration (30 g / l) recorded the highest growth rate.(liquid:31.30 mm and solid:19.60 mm). These results can be used to prevent fungal invasion by not adding any sucrose in the initiation stage.

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