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Using Hydrogen Peroxide for Reducing Bacterial Contamination in Date Palm Tissue Culture

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Abstract: ***The use of hydrogen peroxide as a disinfectant material was evaluated for its effect relevant to bacterial contamination which usually occurs in the micropropagation of date palm. Different concentrations of hydrogen peroxide at different time intervals were examined and compared to ethyl alcohol and distilled water with both of callus and shoot explants of date palm cv. Sewi. The most powerful disinfection with 100% survival occurred by using 10 ppm hydrogen peroxide for 1 min.***
Keywords: "Date palm", "tissue culture", "bacterial contamination", "hydrogen peroxide", "decontaminating agents".

1. Introduction

Date palms are the only species of *Phoenix dactilefra L*. and the family of *Arecaceae* where other species are propagated by seed only (Zaid, 1999). It is worth mentioning that although there are many methods for date palm propagation they are considered in-ideal. The oldest method is propagation by seed that is known as a sexual propagation, the fruits produced are characterized by its inferior quality exhibiting a high level of variation. The second method of propagation is by means of offshoots known as asexual or vegetative propagation. It is true-to-type to the parent palm but are not considered ideal due to the limited number of offshoots borne only during the first 15 years after planting (Jain, 2012).

Recently, the micropropagation technique is preferred due to its several advantages including the production of disease and pest-free cultivars that are genetically identical and true-to-type with the mother plant, in addition to the mass production unlimited by seasonal effects (Kriaa *et al.*, 2011). In spite of these acquired advantages, there are many challenges mainly the microbial infection in the growth media causing losses in most stages (Al-Mussawi, 2010). The presence of these microbes usually results in increased culture mortality but can also results in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Tyagi *et al.*, 2011).

Fungi and bacteria are two different microbial groups associated with date palm tissue cultures (Odutayo *et al.*, 2007, Abass, 2013b) and supported by the medium composition which is a good source of nutrients containing the essential requirements to support their growth and development. Elimination of bacteria requires complex procedures, such as surface sterilization and antibiotic utilization (Leifert and Waits, 1992). Different sources of contamination are present in the preparation and incubation rooms as well as indoor and outdoor air of date palm tissue culture laboratories. For that reason, high contamination rates

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with different species of bacteria occur in most tissue-cultured date palm cultivars at different stages of micropropagation (Abbas, 2013a).

The most serious bacterial contaminant of date palm tissue culture can be isolated from human skin, plant debris as well as the walls and tables of tissue culture laboratories (Odutayo *et al*., 2007).

Different chemical agents and/or antibiotics have been added to the media as prophylactics to control bacterial contamination. Phytotoxicity of the combined treatments is a limiting factor for controlling microbial contamination and recovering healthy plants have to be tested before being added into the culture media (Al-Mayahi *et al.*, 2010).

Hydrogen peroxide (H_2O_2) is a strong oxidizer that forms when water combines with ozone in the atmosphere. The bonds that hold the hydrogen and oxygen atoms together in H_2O_2 are unstable, which causes the molecule to break along the oxygen-oxygen bond, releasing free hydroxyl radicals that serve to oxidize organic matter for control of pathogens.

It is well-known as an antiseptic because of its cytotoxic effects on many bacterial strains (Ikai *et al.*, 2010). The disinfection technique is based on the concept that the hydroxyl radical kills microorganisms as a result of oxidation of cellular components, such as cell membrane, nucleic acid, and other cell components (Shirato *et al.*, 2012). Low concentrations of hydrogen peroxide can be applied in a continuous treatment while misting the hydrogen peroxide solution on the media surface can be used to reduce the microbial load (Larose and Abbot, 1998).

Removing external pollution is of a significant economic return to reduce the loss during micropropagation. From this point of view this research paper aimed to remove/reduce bacterial contamination using hydrogen peroxide in re-sterilization of contaminated tissues which are highly sensitive to many sterilizing compounds with no damage in the tissues or retardation of its growth.

2. Materials and Methods

2.1 Used disinfectant materials:

- Hydrogen peroxide (30% absolute)
- Ethyl alcohol (70% absolute)
- Sterilized distilled water

2.2 Used explants:

- Calli and shoots of Date palm cv. Sewi

The choice of the stages included in this research paper (Callus and Shoot) is critical since they are considered the most important stages of numerical multiplication.

The experiment was conducted in the Central Laboratory of Date Palm Researches and Development, Agriculture Research Center. It is divided into two main parts:

- First: includes studying the effect of various disinfectant materials on bacterial contamination occurs in tissue culture of date palm cv. Sewi.
- Second: includes calculating the survival of explants after the disinfection process.

Hydrogen peroxide and ethyl alcohol were used to control bacterial contamination of explants. Hydrogen peroxide was prepared in different concentration levels (10, 20 and 30 ppm) as shown in figure (1), while ethyl alcohol was used with the concentration (70%) normally used in tissue culture technique. Sterilized distilled water was used as the control treatment. The experiment was repeated on the two different stages of explants; callus and shoot contaminated with bacteria. The contaminated parts were dipped in the disinfectant material (either hydrogen peroxide, ethyl alcohol or water) for different time intervals (1, 3 and 5 minutes) then transferred to a solid medium containing full MS, 30 g/l sucrose, vitamins and;

1. 2,4-D (10 mg/l) + 2ip (3 mg/l) for callus stage
2. BA (0.05 mg/l) + NAA (0.1 mg/l) for shoot stage

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The survival percentage was calculated for each of the two stages with all of the used disinfecting materials at different time intervals using the following equation:

Survival % = No. of survived plants / Total No. of starting plants × 100

Figure 1: Preparation of H_2O_2 concentrations and testing with contaminated shoots

2.3 Statistical analysis:

All treatments used in this study were arranged in a complete randomized block design. Four replicates, three jars in each replicate were used for each treatment and incubated for a week.
The disinfection degree was recorded visually as scores according to Pottino, 1981 as follows:

- Negative results (+) = 1
- Below average results (++) = 2
- Average results (+++) = 3
- Good result (++++) = 4

The obtained data were subjected to analysis of variance and statistically analyzed according to Duncan's multiple range test (Duncan, 1955). The least significant difference (L.S.D) at 0.05 % was calculated.

3. Results and Discussion

3.1. Effect of using different disinfectant materials and treatment time on contamination degree of callus tissues of date palm cv. Sewi

The results as shown in Table (1) indicate that the most effective treatment on the contamination degree of calli of date palm cv. Sewi was achieved by using 10 or 20 ppm hydrogen peroxide for 1 min. which have the same effect as 10ppm hydrogen peroxide for 3 min. while 30 ppm hydrogen peroxide showed the least significant effect followed by 70% alcohol and control water treatments.

Table (1): Effect of using different disinfectant materials and treatment time on disinfection degree of calli tissues of date palm cv. Sewi

Treatment (A)	Period (B)			Mean (A)
	1 min	3 min	5 min	
Water (Control)	1.00	1.00	1.00	**1.00c**
Alcohol (70%)	1.33	1.33	1.00	**1.22c**

H_2O_2 (10 ppm)	4.00	3.67	2.33	**3.33a**
H_2O_2 (20 ppm)	3.67	3.00	3.00	**3.22a**
H_2O_2 (30 ppm)	2.67	2.33	2.00	**2.33b**
Mean (B)	**2.53a**	**2.27a**	**1.87b**	

L.S.D at 0.05% A= 0.4601 B= 0.3564 AB= 0.7969

Means of hydrogen peroxide treatments and time intervals followed with the same letter within each column are not significantly different from each other at 0.05% level.

3.2. Effect of using different disinfectant substances and treatment time on survival of calli tissues of date palm cv. Sewi

Table (2) showed that any of the used concentrations of hydrogen peroxide (10, 20 or 30 ppm) for the least time interval (1 min.) resulted in 100% survival of calli. On the other hand using H_2O_2 with concentration 10 ppm had 100% survival when applied for 3 or 5 min. Survival decreased significantly to 91.67% using 20 ppm hydrogen peroxide for 3 min. followed by 83.33% survival using 20 ppm for 5 min. and 30 ppm for 3 or 5 min. The maximum survival achieved on using 70% alcohol or water was 50% with no significant difference between the two treatments on survival of calli.

Table (2): Effect of using different disinfectant materials and treatment time on survival of calli tissues of date palm cv. Sewi

Treatment (A)	**Period (B)**			**Mean (A)**
	1 min	**3 min**	**5 min**	
Water (Control)	50.00	41.67	33.33	**41.67c**
Alcohol (70%)	41.67	33.33	25.00	**33.33c**
H_2O_2 (10 ppm)	100.00	100.00	100.00	**100.00a**
H_2O_2 (20 ppm)	100.00	91.67	83.33	**91.67ab**
H_2O_2 (30 ppm)	100.00	83.33	83.33	**88.89b**
Mean (B)	**78.33a**	**70.00b**	**65.00b**	

L.S.D at 0.05 A= 9.666 B= 7.487 AB= 16.74

Means of survival and immersion intervals followed with the same letter within each column are not significantly different from each other at 0.05% level.

3.3. Effect of using different disinfectant materials and treatment time on contamination degree of shoot of date palm cv. Sewi

The results in Table (3) showed the effect of using different concentrations of hydrogen peroxide and different time intervals on the disinfection degree of contaminated shoots. The most effective concentration for disinfecting shoot tissues was 10 ppm H_2O_2 for 1 or 3 min. followed by 20 ppm for 1 min. followed by 3 min. or 30 ppm for 1 min. The least effect was for 30% for 3 min. followed by 20 ppm or 30 ppm for 5 min. while no significant effect was noticed for 70% alcohol and water treatments.

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Impact Factor: 6.057
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Table (3): Effect of using different disinfectant materials and treatment time on contamination degree of shoot of date palm cv. Sewi

Treatment (A)	Period (B)			Mean (A)
	1 min	3 min	5 min	
Water (Control)	1.67	1.00	1.00	**1.22c**
Alcohol (70%)	1.67	1.33	1.00	**1.33c**
H_2O_2 (10 ppm)	4.00	4.00	3.33	**3.78a**
H_2O_2 (20 ppm)	3.67	3.33	2.33	**3.11b**
H_2O_2 (30 ppm)	3.33	3.00	2.33	**2.89b**
Mean (B)	**2.87a**	**2.53b**	**2.00c**	

L.S.D at 0.05 A= 0.3765 B= 0.2916 AB= 0.6521

Means of hydrogen peroxide treatments and time intervals followed with the same letter within each column are not significantly different from each other at 0.05% level.

3.4. Effect of using different disinfectant materials and treatment time on survival of shoot of date palm cv. Sewi

The results in Table (4) showed the survival of 100% of shoots achieved with 10% hydrogen peroxide for 1 and 3 min. and decreased to 91.67% with 10 ppm for 5min. and 20 ppm for 1 min. The survival percent also decreased to 83.33 with 20 ppm for 3 min. and 30 ppm for 1 min. and 75% with 20 ppm for 5 min. and 30 ppm for 3 min. while the least significant survival achieved by using 30 ppm hydrogen peroxide for 5 min. was 58.33%. The survival ranged between 33.33-50% in case of using alcohol and water while the lowest survival achieved was 25% when using alcohol or water for 5 min.

Table (4): Effect of using different disinfectant materials and treatment time on survival of shoot of date palm cv. Sewi

Treatment (A)	Period (B)			Mean (A)
	1 min	3 min	5 min	
Water (Control)	50.00	41.67	25.00	**38.89d**
Alcohol (70%)	41.67	33.33	25.00	**33.33d**
H_2O_2 (10 ppm)	100.00	100.00	91.67	**97.22a**
H_2O_2 (20 ppm)	91.67	83.33	75.00	**83.33b**
H_2O_2 (30 ppm)	83.33	75.00	58.33	**72.22c**
Mean (B)	**73.33a**	**66.67a**	**55.00b**	

L.S.D at 0.05 A= 10.13 B= 7.849 AB= 17.55

Means of survival and immersion intervals followed with the same letter within each column are not significantly different from each other at 0.05% level.

From the previous results and as summarized in Tables (5) and (6), we can conclude that there was no significant difference between the disinfection degrees of calli by using 10 or 20 ppm of hydrogen peroxide while there was significant difference in the survival percentage between the two concentrations 10 and 20 ppm. The survival was 100% and 92% respectively. In the shoot stage there was significant difference in

both of the disinfection degree and survival percentage on using both hydrogen peroxide concentrations. The 10 ppm hydrogen peroxide was better for both parameters as shown in figures (2) and (3).

Table (5): Statistical comparison between the mean of disinfection and survival results from callus and shoot stages

Disinfectant material / Conc.	Callus stage		Shoot stage	
	Disinfection	Survival	Disinfection	Survival
H_2O_2 (10 ppm)	3.33a	100a	3.78a	97a
H_2O_2 (20 ppm)	3.22a	92ab	3.11b	83b
H_2O_2 (30 ppm)	2.33b	89b	2.89b	72c
Alcohol (70%)	1.22c	33c	1.33c	33d
Water	1.00c	42c	1.22c	39d

Means within each column followed with the same letter are not significantly different from each other at 0.05% level.

Table (6): Statistical comparison between the mean of immersion interval results from callus and shoot stages

	Callus			Shoot		
	1 min.	3 min.	5 min.	1 min.	3 min.	5 min.
Disinfection	2.53a	2.27a	1.87b	2.87a	2.53b	2.00c
Survival	78a	70a	70b	73a	67a	55b

Means within each column followed with the same letter are not significantly different from each other at 0.05% level.

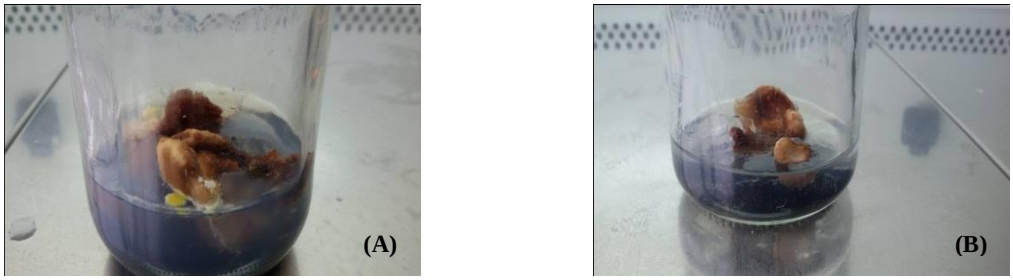

Figure 2: Calli before removal of bacteria (A) and after removal of bacteria (B) using H_2O_2 10 ppm for 1 min

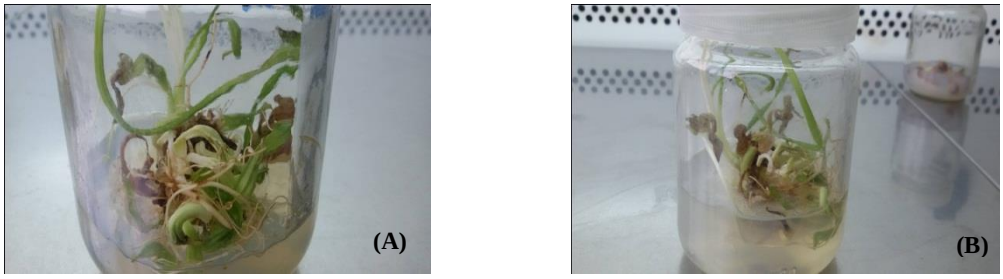

Figure 3: Shoots before removal of bacteria (A) and after removal of bacteria (B) using H_2O_2 10 ppm for 1 min

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It was found also that both alcohol (70%) and water exhibited no significant difference in both disinfection degree and survival percentage.

On the other hand, immersion of the contaminated calli in 10 ppm hydrogen peroxide for 1 min. did not significantly differ than 3 min. in the disinfection degree while for survival they differed significantly. In shoot the opposite was true, there was no significant difference in survival between the two immersion intervals. The 3 and 5 min. differed significantly for disinfection degree and survival in the two tested stages. All results are demonstrated in figure (4).

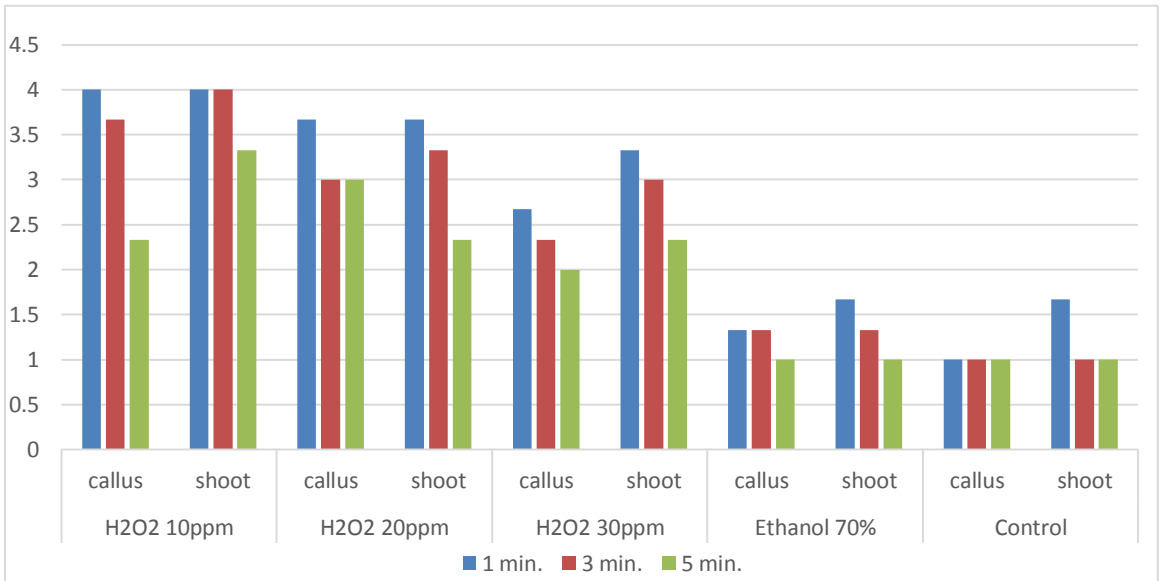

Figure 4: Effect of different H2O2 concentrations, ethanol (70%) and water on disinfection degree of bacterial contamination in callus and shoot stages of Date palm micropropagation

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Hydrogen peroxide is an oxidizing agent that reacts with various microbial components including proteins, lipids and nucleic acids resulting in a loss of structure and function leading to microbial death according to Imlay *et al.,* (1988) while alcohol incompetence may be due to sterilization of plant tissues for a long time because alcohol is an intermediate stage of bacterial fermentation according to Van Waarde *et al*., (1993).

Our results are in accordance with the results of Michelle Finnegan *et al.* 2010 as hydrogen peroxide has been found to be more effective in preventing microbial growth than other oxidative biocides such as chlorine dioxide and peracetic acid which act as antimicrobials decontaminating agents and share the same basic action mechanism of chemical oxidation of cellular components with some differences in their efficacy against microorganisms, while it is not in accordance with Ines Mihaljević *et al.*, 2013 who found that the treatments with hydrogen peroxide showed unsatisfactory results with a high percent of contamination and a low percent of survived explants.

The best results of bacterial disinfection and explant survival obtained with the lowest concentration of hydrogen peroxide can be returned to the fact that oxidizing agents are usually low molecular weight compounds and are considered to pass easily through cell walls to react with internal cellular components leading to cell death. Alternatively, they can severely damage microbial structure causing the release of intracellular components, which are then oxidized (Denyer and Stewart, 1998).

The insignificant effect of 70% alcohol on the disinfection degree in callus and shoot stages is in line with Odutayo *et al.*, 2007 who reported that bacteria species can survive in 96% ethanol for a few hours.

4. Conclusion

Based on our findings, it is recommended to use hydrogen peroxide to disinfect bacterial contamination occurs in Date Palm micropropagation especially in the callus and shoot stages. Using alcohol in disinfection results in the recurrence of bacterial contamination unlike hydrogen peroxide in which release of hydrogen atom has a significant effect in the sterilization procedure of the explants and externally contaminated surfaces. On the other hand, one minute duration at different concentrations is better than five minutes because the lengthy time is desired until the sterilization affects the vitality of the tissue cells. The alcohol also affects the vitality of the callus cells as the alcohol removes the water molecules and the organic solvent substitute compact callus with friable one which means that increasing treatment time increases chemical damage and affects vitality of tissues.

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