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Using Actinomycetes on Controlling Bacterial Contamination of Date Palm During Different Stages *In Vitro*

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Abstract: Bacterial contaminations are considered to be of the most serious constraints facing date palm proliferation *in vitro*. To control date palm *(Phoenix dactylifera*) cv. Sakkoty contaminated by bacteria *in vitro*, actinomycetes (*Streptomyces bobilii* or *S. chloramphenicol*) were studied in these paper. Actinomycetes were studied at the concentrations (0, 50, 100, 250, 500 and 1000 ppm) and added to culture media in different stages of date palm indirect somatic embryogenesis stages (establishment, callus formation, somatic embryos growth, shooting and rooting). It was found that, after twelve weeks, *S. chloramphenicol* was more effective than *S.bobilii* at any concentration used at different stages. Addition of *S. chloramphenicol* at the concentration of 500 ppm to the culture media during all studied stages was the prefer treatment which caused the best number of explants free contamination, the best number of survived explants, the highest number of embryos and the best degree of growth vigor during growth and development of explants.

Key words: *Phoenix dactylifera* · Contamination · Actinomycetes

INTRODUCION

In vitro multiplication has been developed and applied to plant improvement as well as the production of planting materials in crops generally. In date palm *(Phoenix dactylifera*) in particular, the use of tissue culture techniques for the production of planting materials has been in practice for many years, in different parts of the world [1-3]. Several proplems are still associated with the micropropagation of date palm; probably the most important being explant contamination, browning and embryogenesis iniation [4]. The concept of propagation, on agar, of plant tissues from fast growing tissue such as apical meristems has been well established for many decades. In the same way as virologists incorporate antimicrobials into their tissue culture media to control bacterial growth, so plant propagators have included such agents in their agar media [5]. Leary *et al*. [6] found that, pure cultures of a Gram-, rod-shaped bacterium, which produced endospores after 3-5d on solid medium, were isolated exclusively from date palm tissue cultures Electron microscope examination of thin sections of the bacteria revealed the bilayer membrane typical of Gram-bacteria and confirmed the nature of the spores as true endospores. Biochemical and physiological tests identified the bacteria as *Bacillus circulans*. *Bacillus circulans* was consistently isolated from the internals tissues, including the meristem, of apparently healthy offshoots of date palm. When meristem and embryo callus tissue culture samples were injected with isolates from similar tissue culture samples and from offshoots, most produced a rapid, destructive soft rot of the tissues. Also, Leary and Chun [7] stated that, when inculcated to seeds of 8date palm cultivars, *Bacillus circulans* isolate B02-3 pathogenic to date palm tissue cultures, significantly reduced the germination frequency of 3 cultivars and the FW and lengths of seedlings of 4 cultivars. A significant number of inoculated seedlings of these 4 cultivars showed disease symptoms consisting of necrosis that progressed down the cotyledon, followed by wilting.

Therfore micro-organisms were isolated from soil and rhizosphere of crops which exhibited reductions disease. Some other isolates of fungi, actino and bacteria exhibited low effect on white rot disease incidence in pots. Isolates obtained from soil and rhizoshphere some white rot inhibitory crops were tested for antagonism with *Sclerotium cepivorum* on media. Some of these isolates

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exhibited wide inhibition zones which due to specific toxic metabolites externally excreted by the bio-agent to inhibit mycelial growth of the causal pathogen. Such type of antagonism was reported by many researchers [8-11]. Sabaou and Bouaga [12] isolated *Nocardiopsis dassonvillei* and 1 of *Streptomyces alni* displayed the same mode of action in parasitizing Fusarium. In an autoclaved palm-grove soil, both actinomycetes strongly reduced propagule populations of *F. sp* a pathogen of date palm. This action was less pronounced in the same nonautoclaved soil, where the antagonism of actinomycetes was extenuated by the presence of other soil microorganisms. However, both *N. dassonvillei* and particularly *S. alni* decreased the population of *F. sp*. [13] stated that, culturing filaterates of isolates from soil, rhizosphere and crops were toxic to mycelial growth of *Scherotium cepivum*. Therefore tests of antagonism were continued with garlic clove treatments with bio-agent spores and culture filterates. Promising results were obtained with these treatments which could be recommended for the biological control of garlic white rot disease. The most effective isolates tested were the fungus and the actinomycetes *Streptomyces bobilii* and *S. grisiobrunneus* which were mostly without damage to seed germination of many vegetable and field crops tested.

To mange date palm *(Phoenix dactylifera*) cv. Sakkoty contamination *in vitro* by bacteria, actinomycetes *(Streptomyces chloramphenicol* or *S. bobilii*) were studied at different concentrations in starting, callus, embryos, shooting and rooting stages of date palm somatic embryos protocol.

MATERIALS AND METHODS

The present study was performed throughout the period from 2004-2007 at the Central Laboratory of Date Palm Researches and Development Giza, Egypt.

Date palm explants establishment frequently requires special procedures to escape or avoid problems that are associated with contaminations. Date palm culture suffers habitual concentration problems. The contaminants were internal and occurred despite the method of sterilization. The occurrence of contamination days, weeks or even months after culture establishment on nutrient medium has been reported [14-17].

For the previews reason, the following experiments were done. Explants of date palm cv. Sakkoty contaminated with bacteria were collected from different stages of date palm somatic embryo formation protocol (starting, callus, embryo, soot and root) grow on the Central Laboratory of Date Palm Researches and Development.

Actinomycetes *S. chloramphenicol* (pharmacy) and *Streptomyces bobilii* isolated by {Mr. Abd El-Rahman Metwally researcher in Central Laboratory of Date Palm Researches and Development Giza, Egypt from soil according to Tuite [18] and then grown on Starch nitrate medium consisted of g/l; KNO_3 2.0+ Starch 20+ $MgSO_4.7H_2O$ 0.5+ $K_2HPO_4.3\ H_2O$ 1.0+ NaCl 0.5+ agar agar 15.0+ 1ml of trace element in solution consisted of 0.1g/l $FeSO_4$; $MnCl_2.7\ H_2O$ and $ZnSO_4 7\ H_2O$. for 3weeks and then filtered asceptically into sterile Wat.1 double filter paper and filtrates were received in autoclaved flasks} were added at concentrations 0, 50, 100, 250, 500 and 1000 ppm to different culture media at different stages under investigation. All different media were autoclaved for 20 min. at 121°C (1.2 Kg/cm^2). Media of each treatment were distributed in jars 150 ml at rate of 35 ml/jar.

Explants Type and Culture Media: Sub-shoot tip sections which cultured on media consisted of MS+ 10 ppm 2,4-D (dichlorophenoxy acetic acid)+ 3 ppm 2iP(6-γ,γ-Dimethylallylamino purine) + 40 ppm adenine sulphate $2H_2O$ + 170 ppm glutamine+ 3g/l activated charcoal + 30 sucrose and 6 g/l agar used as explants on stage (1). 1×1 cm friable callus which cultured on media consisted of MS+ 10 ppm 2,4-D + 5 ppm NAA (naphthalene acetic acid)+ 3 ppm 2iP+ 1.5g/l activated charcoal + 30 sucrose and 6 g/l agar as explants to stage (2). Clusters contained 2-4 embryos as explants which cultured on media consisted of MS+ 0.2 ppm 2iP + 0.1 ppm NAA 1.5g/l activated charcoal + 30 sucrose and 6 g/l agar to stage (3). Clusters contained 4-5 shoots which cultured on media consisted of MS+ 3 ppm 2iP + 30 sucrose and 6 g/l agar stage (4). Shoot of about 8 cm in length with 2-3 leaves which cultured on media consisted of MS+ 0.1 ppm NAA+ 3g/l activated charcoal + 30 sucrose and 6 g/l agar used as plant materials on stage (5).

Each treatment contained 12 jars (12 replicates), which were incubated in a growth room at 26±2°C under darkness in stage (1) and (2) but in stage (3, 4 and 5) at 16 hr illumination of 2000 lux (white fluorescent lamps). Subculturing the explants was done onto the same medium every 3weeks and 3subcultures were done on each stage.

Data Were Taken as Follows: Number of plants free contamination, number of survived plants at all experiments and number of embryos on stage (3) was recorded. Data obtained were subjected to the analysis of variances of randomized complete design. LSD at 5% level of significance was used to compare between means [19]. Growth vigor's on stages (4 and 5) were estimated visually as follows:

Negative result (-) =1 Below average results (+) = 2
Average results (++) = 3 Good results (+++) = 4
According to Pottino [20]

RESULTS

Establishment Stage: The effects of different concentrations of *Streptomyces chloramphenicol* and *S. bobilii* on date palm *(Phoenix dactylifera*) cv. Sakkoty *in vitro* number of contaminated explants and survived explants after 12weeks from culturing during starting stage are presented in Table 1. Data in Table 1 revealed that the number of explants free contamination and survived explants as affected by *S. chloramphenicol* and *S. bobilii* different concentrations. It's obvious that using *S. chloramphenicol* was more effective for producing higher number of explants free contamination than *S. bobilii* as the results were (5.2 and 2.0) respectively. Concerning the effect of concentration of actinomycetes regardless the type of actinomycetes, it was found that, the highest number of explants free contamination (6 explants) produced by using 1000 ppm *S.chloramphenicol* followed by using 500 ppm which gave (5.5) with no significant deference's in between.

Concerning the effect of interaction between treatments and actinomycetes data clearly indicated that, using *S. chloramphenicol* at the concentrations 500 or 1000 ppm were the best treatments which gave the highest number of explants free contamination (8.0explants).

On the other hand, data in Table 1 showed a highly significant number of survived explants by using *S. chloramphenicol* than *S. bobilii*. Using actinomycetes at 100 or 250 gave the highest significant values of survived explants (8.0).

As regards the effects of the interaction between actinomycetes types and concentrations on survived explants, it was found that, using *S.chloramphenicol* at the concentrations of (250 or 500 ppm) on Sakkoty explants gave the highest significant number of survived explants (10).

Callus Formation Stage: Data in Table 2 revealed that, number of explants free contaminated by bacteria and survived explants through callus formation stage were affected by type and concentrations of actinomycetes after 12weeks from culturing explants during callus formation stage.

It appears from this Table that, the highest number of explants free contamination (8explants) recorded by using *S. chloramphenicol* at the concentration 500 or 1000 ppm whereas, the highest number of survived explants (9) recorded as the results of culturing explants at concentration 100 or 250 ppm followed by 500 ppm with significant differences between them.

Respecting to the effect of different actinomycetes concentrations means number revealed that raising the concentrations of tested actinomycetes in culture media gradually from 0.0 to 1000 ppm raising significantly the number of explants free contamination from 0.0 to 6 explants.

Concerning the effect of actinomycetes type, data in Table 2 shows that addition of *S. chloramphenicol* which gave 5.3 explants free contamination and 7.5 survived explants to culture media was more effective than *S. bobilii* which gave 2.0 explants free contamination and 5.8 srvived explants with significant deference's in between.

In conclusion, it appears that using *S. chloramphenicol* at the concentration 500 ppm on Sakkoty explants was the prefer concentration during callus formation stage which gave the highest number of explants free contamination (8explants) and gave (8) survived explants.

Somatic Embryogenesis Stage: The effects of different concentrations of actinomycetes on explants free contamination, survived explants and number of embryos on somatic embryogenesis stage are presented in Table 3. Data clearly indicated that, raising the concentrations of actinomycetes in culture media gradually from 0.0 to 1000 ppm raising significantly the number of explants free contamination from 0.0 to 6.5 explants.

Concerning the effect of actinomycetes type, data indicated that using *Streptomyces chloramphenicol* which gave 5.3 explants free contamination, 8survived explants and 2.5 embryos/explant was more effective than *S. bobilii* which gave 2.0 explants free contamination, 6.5 survived explants and 0.8 embryos/explant with significant deference's in between.

Table 1: The effects of *Streptomyces chloramphenicol* and *S.bobilii* on *Phoenix dactylifera* cv. Sakkoty explants free contamination and survived explants after 12weeks on starting stage

Treatnents(p.p.m)		*S.chloramphenicol*	*S. bobilii*	Mean (B)
Explants free contamination	Cont.0	0.0	0.0	0.0
	50	3.0	1.0	2.0
	100	5.0	2.0	3.5
	250	7.0	2.0	4.5
	500	8.0	3.0	5.5
	1000	8.0	4.0	6.0
	Mean (A)	5.2	2.0	
Survived explants	Cont.0	6.0	6.0	6.0
	50	7.0	6.0	6.5
	100	9.0	7.0	8.0
	250	10.0	6.0	8.0
	500	10.0	5.0	7.5
	1000	7.0	4.0	5.5
	Mean (A)	8.2	5.7	
L.S.D for explants free contamination		(A)=1.021	(B)=0.5894	(AB)=0.7219
L.S.D for survived explants		(A)=1.193	(B)=0.6885	(AB)=0.8433

Table 2: The effects of *Streptomyces chloramphenicol* and *S. bobilii* on *Phoenix dactylifera* cv. Sakkoty explants free contamination after 12weeks on callus formation stage

Treatnents(p.p.m)		*S.chloramphenicol*	*S. bobilii*	Mean (B)
Explants free contamination	Cont.0	0.0	0.0	0.0
	50	4.0	0.0	2.0
	100	5.0	2.0	3.5
	250	7.0	2.0	4.5
	500	8.0	4.0	6.0
	1000	8.0	4.0	6.0
	Mean (A)	5.3	2.0	
Survived explants	Cont.0	5.0	5.0	5.0
	50	7.0	6.0	6.5
	100	9.0	7.0	8.0
	250	9.0	7.0	8.0
	500	8.0	6.0	7.0
	1000	7.0	4.0	5.5
	Mean (A)	7.5	5.8	
L.S.D for explants free contamination		(A)=1.078	(B)=0.6225	(AB)=0.7625
L.S.D for survived explants		(A)=1.156	(B)=0.6674	(AB)=0.8174

Table 3: The effects of *Streptomyces chloramphenicol* and *S. bobilii* on *Phoenix dactylifera* cv. Sakkoty explants free contamination, survived explants and number of embryos after 12weeks on embryogenesis

Treatnents(p.p.m)		*S.chloramphenicol*	*S. bobilii*	Mean (B)
Explants free contamination	Cont.0	0.0	0.0	0.0
	50	3.0	0.0	1.5
	100	6.0	2.0	4.0
	250	6.0	3.0	4.5
	500	8.0	3.0	5.5
	1000	9.0	4.0	6.5
	Mean (A)	5.3	2.0	
Survived explants	Cont.0	6.0	6.0	6.0
	50	7.0	6.0	6.5
	100	8.0	7.0	7.5
	250	10.0	8.0	9.0
	500	9.0	8.0	8.5
	1000	8.0	4.0	6.0
	Mean (A)	8.0	6.5	
Number of embryos	Cont.0	0.0	0.0	0.0
	50	2.0	0.0	1.0
	100	3.0	2.0	2.5
	250	3.0	2.0	2.5
	500	5.0	1.0	3.0
	1000	2.0	0.0	1.0
	Mean (A)	2.5	0.8	
L.S.D for explants free contamination		(A)=1.078	(B)=0.6225	(AB)=0.7625
L.S.D for survived explants		(A)=1.127	(B)=0.6507	(AB)=0.7969
L.S.D for number of embryos		(A)=0.8659	(B)=0.4999	(AB)=0.6123

Table 4: The effects of *Streptomyces chloramphenicol* and *S.bobilii* on *Phoenix dactylifera* cv. Sakkoty explants free contamination, survived explants and growth vigors after 12weeks on proliferation stage

Treatnents (p.p.m)		*S.chloramphenicol*	*S. bobilii*	Mean (B)
Explants free contamination	Cont.0	0.0	0.0	0.0
	50	4.0	1.0	2.5
	100	5.0	3.0	4.0
	250	7.0	3.0	5.0
	500	10.0	4.0	7.0
	1000	10.0	4.0	7.0
	Mean (A)	6.0	2.5	
Survived explants	Cont.0	5.0	5.0	5.0
	50	7.0	5.0	6.0
	100	9.0	7.0	8.0
	250	9.0	6.0	7.5
	500	10.0	6.0	8.0
	1000	7.0	4.0	5.5
	Mean (A)	7.8	5.5	
Growth vigors	Cont.0	1.0	1.0	1.0
	50	2.3	1.7	1.9
	100	3.0	2.0	2.5
	250	3.3	2.3	2.8
	500	3.0	2.0	2.5
	1000	2.0	1.0	1.5
	Mean (A)	2.4	1.6	
L.S.D for explants free contamination		(A)=1.047	(B)=0.6046	(AB)=0.7405
L.S.D for survived explants		(A)=1.137	(B)=0.6565	(AB)=0.8041
L.S.D for growth vigors		(A)=0.6569	(B)=0.3793	(AB)=0.4645

Table 5: The effects of *Streptomyces chloramphenicol* and *S.bobilii* on *Phoenix dactylifera* cv. Sakkoty explants free contamination, survived explants and growth vigors after 12weeks on rooting formation stage

Treatnents(p.p.m)		*S.chloramphenicol*	*S. bobilii*	Mean (B)
Explants free contamination	Cont.0	0.0	0.0	0.0
	50	3.0	1.0	2.0
	100	5.0	2.0	3.5
	250	8.0	2.0	5.0
	500	8.0	3.0	5.5
	1000	10.0	4.0	7.0
	Mean (A)	5.7	2.0	
Survived explants	Cont.0	5.0	5.0	5.0
	50	7.0	7.0	7.0
	100	8.0	7.0	7.5
	250	10.0	8.0	9.0
	500	10.0	9.0	9.5
	1000	6.0	7.0	6.5
	Mean (A)	7.7	7.2	
Growth vigors	Cont.0	1.0	1.0	1.0
	50	1.7	1.3	1.4
	100	1.7	1.7	1.7
	250	2.0	1.7	1.8
	500	1.7	1.3	1.6
	1000	1.3	1.0	1.2
	Mean (A)	1.5	1.3	
L.S.D for explants free contamination		(A)=1.078	(B)=0.6225	(AB)=0.7625
L.S.D for survived explants		(A)=1.156	(B)=0.6674	(AB)=0.8174
L.S.D for growth vigors		(A)=0.5866	(B)=0.3387	(AB)=0.4148

As regards the effects of the interaction between actinomycetes types and concentrations it's evident that, the best concentration used 500 ppm from *S.chloramphenicol* on Sakkoty explants which gave 8.0explants free contamination, 9.0survived explants and 5.0embryos/explant compared with the other concentration and type.

Proliferation Stage: As soon as, the effect of interaction between actinomycetes type and different concentration used on the proliferation of date palm cv. Sakkoty during shooting stage data in Table 4 showed that, supplementation of *S. chloramphenicol* at the concentration (500 ppm) to the culture medium gave the highest number of explants free contamination

Callus formation Embryo formation Shoot formation Root formation

Fig. 1: Different explants at different stages cultured on medium with 500mg/l *S.chloramphenicol*

(10.0 explants) and the highest number of survived explants (10.0 explants).

Meanwhile the addition of *S. chloramphenicol* at the concentration (250 ppm) to the culture medium gave the highest degree of growth vigors (3.3) followed by (3) which recorded as the results of addition *S. chloramphenicol* at the concentration (100 or 500 ppm) with no significant differences among them.

Generally, the addition of actinomycetes to the culture media from concentration (50 to500 ppm) significantly increases the number of explants contamination (2.5 to7 explants), survived explants (6 to 8) and the degree of growth vigors (1.9 to 2.5) compared with the control which gave the lost number of explants free contamination (0explants), the lowest number of survived explants (5explants) and the lowest degree of growth vigor's (1.0).

Rooting Stage: Concerning the interaction between actinomycetes type and concentration it was found that, the prefer treatments in Table 5 which gave the highest number of survived explants (10 explants) in Sakkoty and 8.0 explants free contamination and 1.7 degree of growth vigor's was 500 ppm *S.chloramphenicol*.

This study shows that, using actinomycetes on *Phoenix dactylifera* cv. Sakkoty contaminated explants on rooting stage at any concentration used significantly increased explants free contamination, survived plants and degree of growth vigor as compared to control media.

Regarding actinomycetes type, date clearly indicated that the addition of *S. chloramphenicol* significantly increased explants free contamination (5.7) compared with *S. bobilii* (2.0) and no significantly increased the number of survived explants (7.7) and the degree of growth vigors (1.5) compared with *S.bobilii* which gave (7.2) survived explants and (1.3) degree of growth vigors.

Contamination, the best number of survived explants, the highest number of embryos and the best degree of growth vigor during growth and development of explants Figure 1.

DISCUSSION

From the above results we can concluded that, addition of *S. chloramphenicol* at the concentration 500 ppm on the culture media during establishment, callus, somatic embryo, shooting and rooting stages on *Phoenix dactylifera* cv. Sakkoty contaminated explants was the prefer treatment which caused the best number of explants free contamination combined with the best number of survived explants above to the highest number of embryos and the best degree of growth vigor on growth and development of explants. These results were agreement with Niedz, randall and Bausher, Michael [21], shows that before plant cell tissue, or organ cultures can be established for commercial or research purposes it is necessary to remove bacteria and fungi. Eliminating bacteria and fungi from plant tissue derived from greenhouse or field sources can be extremely difficult.

Antibiotics are used on again a process that must have certain consequences. It is not surprising that, since the 1950s, antibiotic resistance has emerged amongst the plant pathogens and that the industry has been forced to seek alternative approaches [22]. Administration by injection of oxytetracycline to fruiting and ornamental palm trees has been used successfully for some time in Florida [22]. The antibiotics least likely to be phytotoxic are those acting at sites such as the bacterial cell wall rather than those which act on the ribosomes or DNA. Mycoplasmas [23] and possibly L-forms [24] may cause significant contamination, so β-lactams are not whole answer.

Results under investigation recorded that, actinomycetes used in this study (*S. chloramphenicol*. or *S. bobilii*) at different concentrations enhanced the number of explants free contamination in different stage of date palm somatic embryo formation protocol compared with control medium without actinomycetes. In this respect, Abd-El Kareim *et al*. [25] stated that, on *Phoenix dactylifera* cv. Zaglol culturing *in vitro* using streptomycin at the concentration 250 ppm was more effective in increased survival percentage and decreased the degree of contaminated explants by bacteria on starting, maturation & germination and multiplication Streptomycin selects resistant bacteria which are resistant to other antibiotics [22] and there is some measure of cross-resistance to other aminoglycosides. The relative ease with which certain important human pathogens may become dependent on streptomycin is also a matter for concern [26]. It should not go unnoticed that a decrease in the clinical use of streptomycin and tetracycline in Denmark has corresponded with the decline of one strain of methicillin-resistant *Staphylococcus aureus* which was also resistant to these two drugs [27].

S. chloramphenicol. was superior than *S. bobilii* Chloramphenicol is a broad spectrum antibiotic with bacteriostatic activity however, it may be bactericidal in high concentrations or when used against highly susceptible organisms. The mechanism of action of Chloramphenicol is by inhibition of bacterial protein synthesis in susceptible organisms. The antimicrobial activity of Chloramphenicol covers a wide range of bacteria. *Streptomyces gresies* (waksman) was records as a biocontrol agent to control root rots [28].

Results under discussion revealed that, raising the concentration of actinomycetes in culture medium at different stages above 500p.p.m decreased the number of survived explants, growth vigor and number of new formed embryos. In this respect, Abd-El Kareim *et al*. [25] stated that, on maturation & germination and multiplication stages of date palm somatic embryos formation treated explants by Streptomysin at the concentration 50 ppm gave the highest degree of growth vigor and the lowest degree of browning compared with the higher concentrations.

Treatments that are often effective in removing contaminants are also toxic to the plant tissue. Or, contaminants may be effectively removed from the surface of the plant pieces, but internal contaminants (if present) will remain and will eventual grow and ruin the cultures.

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