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Full Length Research Paper

Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization

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***Lilium candidum* L. is a species which grows in the South West Anatolia region of Turkey. It is a bulbous plant with beautifully scented flowers and is used in the floral industry. The bulbs are produced by using traditional propagation and *in vitro* techniques. Micropropagation is a rapid propagation technique, but the greatest problem is contamination with fungi and bacteria. Antibiotic and fungicide treatments were done after sterilization for micropropagation of *L. candidum*. Fungal contaminants formed during the culture were determined. Bulb scales were used as explants (5 - 10 mm width) and were cultured in photoperiodic conditions (16 h light, 8 h dark) or complete darkness. Bulb scales rinsed in water were surface sterilized, then solutions containing chemotherapeutic substances (Benomyl, Nystatin, Streptomycin, Penicillin) in different combinations were applied for 30 min and subsequently were cultured in MS medium with supplement 0.1 mg dm^{-3} NAA + 0.01 mg dm^{-3} BA. During the experiment, fungal contaminants were observed in full treatments. Determined contaminants were identified according to their morphological and cultural characteristics by cultivation and were comprised of: *Fusarium, Penicillium, Alternaria, Rhizopus, Cylindrocarpon* and *Aspergillus* species. The most effective treatment against fungal contaminations was achieved by utilizing a Benomyl (100 mg dm^{-3}) + Nystatin (100 mg dm^{-3}) treatment combination.**

Key words: Contaminating microorganisms, *Lilium candidum*, bulb scale, *in vitro* culture, treatment, antibiotic, fungicide.

INTRODUCTION

Lilium are bulbous plants with many different species that have a special importance as cut flowers among ornamental plants (Uzun, 1984) and are economically important due to their attractive flowers (Nhut, 1998). Traditional production is carried out with bulbils, bulblets and bulbs. It is also possible to produce plants by tissue culture techniques. With the micropropagation technique, a large number of plants can be propagated in a very short time. Micropropagation has been widely used as an area of biotechnology for the commercial production of ornamental plants (Bürün et al., 2001).

Even though it is possible to produce a large number of plants by micropropagation, the greatest problem in this technique is contamination. A wide range of microrganisms (filamentous fungi, yeasts, bacteria, viruses and viroids) and micro-arthropods (mites and thrips) have been identified as contaminants in plant tissue cultures. Contaminants may be introduced with the explant, during manipulations in the laboratory, by micro-arthropod vectors (Tanprasert and Reed, 1997; Leifert and Cassells, 2001) or endophytic bacteria (Reed et al., 1995; Pereira et al., 2003). Fungus may arrive with an explant, or airborne, or enter a culture (Babaoğlu et al., 2001). Frequently encountered bacterial and fungal contaminations especially in laboratories of commercial micro-

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Abbreviations: MS, Murashige and Skoog; **BA,** benzyladenine; **NAA,** naphthalene acetic acid.

propagation pose a considerable problem (Reed et al., 1998). Studies on the effect of antibiotics and fungicides on these kinds of contaminants have been carried out (George, 1993).

Shields et al. (1984) analysed the effects of a number of fungicides against *in vitro* fungal contaminants and their toxicity in tobacco cultures. They recommend 2 fungicides, carbendazim and fenbendazole (30 µg cm^{-3}). In addition imizalil (20 µg cm^{-3}) and captofol (100 µg cm^{-3}) were alternative fungicides to prevent fungal contamination and a mixture of propiconazole plus carbendazim was effective to control fungal contaminants (George, 1993).

Wittenbach and Bukavoc (1980) reported that benomyl (100 µg cm^{-3}) reduced the contamination of cherry fruits without affecting fruit growth unfavourably (George, 1993).

It has been stated that not single but combinatorial use of antibiotics has shown synergistic effects in both control of microorganizms and reduction of plant damage. Because most antibiotics have a narrow target spectrum for bacteria, it is typical that they be used in an ordered or step-by-step fashion. It has been determined that 10 µg cm^{-3} Rifampin + 1 g dm^{-3} Benomyl has been an important effective combination in the control of fungal and bacterial contaminants in *Camellia* cultures (Haldeman et al., 1987).

Reed et al. (1998) had observed internal bacterial contamination in hazelnut shoot cultures and contaminants were evident at culture establishment, or became apparent after several subcultures. They had treated plant material with antibiotics timentin + streptomycin or gentamycin + streptomycin and determined that the antibiotics combination was more effective, where no single antibiotic was effective for all bacterial isolates from hazelnut shoot cultures.

Kubota and Tadokoro (1999) examined photoautotrophic (sugar-free) micropropagation for many different plant species. One of the advantages of photoautotrophic micropropagation is the low risk of contamination and addition of $AgNO_3$ in the medium suppressed growth of nonpathogenic contaminants without reducing fresh and dry weight, and number of leaves of tomato plantlets.

In this study, the identification of fungal contamination emerging during micropropagation of *Lilium candidum* and the effects of antibiotic and fungicide treatments after sterilization was determined.

MATERIALS AND METHODS

Plant material and nutrition medium for micropropagation

In the micropropagation of species *Lilium,* bulb scales and leaves have been successfully used as explants (Mansuroğlu and Gürel, 2001). The *L. candidum* bulbs were collected from Dalyan - Muğla. Each bulb-scale was cut into 5-10 mm pieces and each part excised was placed into a culture tube. For each treatment, 40 explants (40 culture tubes) were used.

Medium was prepared and used in the following mixture: 30 g dm^{-3} sugar, 8 g dm^{-3} agar was added to MS medium (Murashige-Skoog, 1962) containing 0.1 mg dm^{-3} NAA + 0.01 mg dm^{-3} BA plant growth regulator were prepared according to Franklin and Dixon (1994) and pH adjusted to 5.7 before autoclaving for 15 min. The sterilization of fungus identification media was made in the same way by autoclaving.

Isolation and identification of filamentous microfungi

During micropropagation, fungal contaminants were transferred into tubes containing potato dextrose agar (PDA, Difco 0013) and were kept at +4°C for identification.

The Fungi were later inoculated on PDA (Difco 0013), malt extract agar (Difco 0112) and yeast and mould agar (Oxoid CM920). Plates were incubated in the dark at laboratory temperature (25°C) for 5-15 d and the microscopic fungi were identified using the diagnostic keys of Hasenekoğlu (1991) and Barnett and Hunter (1998). For identification purposes, slide cultures were prepared on malt extract agar and stained with lactophenol-blue.

Surface sterilization of plant material and treatments of chemotherapeutic substances

Lilium bulb scales were rinsed in tap water. They were dipped in 96% ethanol for 2 min, then in 2.25% Na–hypochlorite solution, with one drop of 0.1% Tween 80 for 20 min and rinsed 4 times in sterile distilled water.

Lilium bulb scales were treated with solutions containing antibiotics and fungicides in different combinations for 30 min after surface sterilization of bulb scales. These combinations are as follows:

Treatment 1. Streptomycin 200 mg dm^{-3} + Penicillin 200 mg dm^{-3}
Treatment 2. Streptomycin 400mg dm^{-3} + Penicillin 400 mg dm^{-3}
Treatment 3. Benomyl 50 mg dm^{-3} + Nystatin 50 mg dm^{-3}
Treatment 4. Benomyl 100 mg dm^{-3} + Nystatin 100 mg dm^{-3}
Treatment 5. Streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3}
Treatment 6. Streptomycin 600 mg dm^{-3} + Benomyl 150 mg dm^{-3}

The test of phytotoxicity

In the trials, it was analyzed that superface had or not phytotoxicity on the plant of secondary metabolites that were reproduced during the development of fungal contaminants that were isolated. For that reason, contaminating products were sown in a medium (Saboraund Dextrose Broth) and they were applied on *Lolium perenne* seeds, as *Lolium* normally germinate in a very short time. The treated seed were tested for germination and phytotoxicity in the isolates was assessed.

The conditions of culture room

Culture room temperature was 23°C, with a photoperiod of 16 h light (1600 lux), 8 h dark and total darkness. Development of the bulblets from explants was observed in the culture room.

RESULTS

Fungal contaminants were identified during micropropagation of *L. candidum.* The fungal contaminants found are listed in Table 1 according to fungicide and antibiotic

Table 1. The list of fungal species that were determined in accordance with their applications.

Treatment[a] and environmental conditions[b]	Determined fungal contaminations[c]															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Photoperiodic conditions																
Control			x		x											
T1			x											x		x
T2									x							x
T3																x
T4	x															x
T5													x			x
T6			x													x
Dark Conditions																
Control																
T1			x						x		x					
T2		x							x			x				x
T3		x												x		x
T4			x						x	x	x					x
T5		x	x	x		x	x							x		x
T6		x						x	x	x						x

[a]: Treatment
Control
T1: Streptomycin (200 mg dm^{-3}) + Penicillin (200 mg dm^{-3})
T2:Streptomycin (400 mg dm^{-3}) + Penicillin (400 mg dm^{-3})
T3: Benomyl (50 mg dm^{-3}) + Nystatin (50 mg dm^{-3})
T4: Benomyl (100 mg dm^{-3}) + Nystatin (100 mg dm^{-3})
T5: Streptomycin (300 mg dm^{-3}) + Benomyl (75 mg dm^{-3})
T6: Streptomycin (600 mg dm^{-3}) + Benomyl (150 mg dm^{-3})
[b]: Environmental conditions
Photoperiodic conditions (16 hours day, 8 hours darkness).
Totally dark conditions
[c]: Fungal contaminants
1: *Alternaria* sp.; 2: *Aspergillus* sp.; 3: *Aspergillus niger* ; 4.*Cylindrocarpon* sp.; 5. *Fusarium* sp.; 6: *Penicillium* sp.1;
7: *Penicillium* sp. 2; 8: *Penicillium* sp. 3; 9: *Penicillium* sp. 4; 10: *Rhizopus* sp.; 11: *Rhizopus stolonifer;* 12: *Mycellia sterilia*
Unidentified isolates:
13: Colony type 1; 14: Colony type 2; 15: Colony type 3; 16: Colony type 4.

treatments. In the cultured photoperiodic condition (16 h light, 8 h dark) a total of 29 contamined cultures were identified (10.43% of cultured explants) and the fungal agent, *Aspergillus niger, Penicillium* sp. 4, *Alternaria* sp. and *Fusarium* sp. were identified. In experiments using antibiotic/fungicide treatments, Treatment 1, Treatment 6 and control, the contaminants were observed as *Aspergillus niger* and formed 10.34% of total contamination. The fungus that caused contamination in Treatment 2 was identified as *Penicillium* sp. 4 and formed 3.44% of total contamination. The fungus that caused contamination in Treatment 4 (Benomyl 100 mg dm^{-3} + Nystatin 100 mg dm^{-3}) was identified as *Alternaria* sp. and formed the same rate of contamination as *Penicillium.* The rest of contaminations were unknown and these unidentified isolates were classified under four colony types in both the photoperiodic growth condition and darkness growth condition (but Colony type 3 was noticed only in the darkness growth condition). Colony type 1 (3.44% of total contamination, in Treatment 5), Colony type 2 (the same rate of contamination as Colony type 1, in Treatment 1), and Colony type 4 (68.9% of total contamination, in all of the treatments). The contami-nations in controls were identified as *Fusarium* sp. and formed 6.89% of total contamination. The most contami-nants were observed in Treatment 1 (Streptomycin 200 mg dm^{-3} + Penicillin 200 mg dm^{-3}), Treatment 2 (Strepto-mycin 400 mg dm^{-3} + Penicillin 400 mg dm^{-3}), Treatment 3 (Benomyl 50 mg dm^{-3} + Nystatin 50 mg dm^{-3}) and Treatment 5 (Streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3}).

In dark-grown culture, a total of 46 contaminated tubes were observed (16.60% contaminations in this experiment) including *Penicillium* sp *(1, 2, 3, 4), Aspergillus* sp. and *A. niger,* Rhizopus sp., *R. stolonifer* and *Cylindrocarpon* sp. *Penicillium* sp. 1 (2.17% of total contamination) and *Penicillium* sp. 2 (2.17% of total contamination) were observed only in streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3} treatments (in treatment). *Penicillium* sp. 3 (2.17% of total contamination) were observed only in Treatment 6 (Streptomycin 600 mg dm^{-3} +

Benomyl 150 mg dm^{-3}). The fungi that causes contamination in Treatment 4 (Benomyl 100 mg dm^{-3} + Nystatin 100 mg dm^{-3}), Treatment 6 (Streptomycin 600 mg dm^{-3} + Benomyl 150 mg dm^{-3}) and both of Streptomycin + Penicillin treatments (Treatments 1, 2) were identified as *Penicillium* sp. 4 and formed 17.39% of total contamination. Contaminants in Treatment 1 (Streptomycin 200 mg dm^{-3} + Penicillin 200 mg dm^{-3}), Treatment 4 (Benomyl 100 mg dm^{-3} + Nystatin 100 mg dm^{-3}) and Treatment 5 (Streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3}) were identified as *Aspergillus niger* and comprised 17.39% of total contamination. Contaminants in Treatment 2 (Streptomycin 400 mg dm^{-3} + Penicillin 400 mg dm^{-3}), Treatment 3 (Benomyl 50 mg dm^{-3} + Nystatin 50 mg dm^{-3}), Treatment 5 (Streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3}) and Treatment 6 (Streptomycin 600 mg dm^{-3} + Benomyl 150 mg dm^{-3}) were identified as *Aspergillus* sp. and formed the same rate of contamination as *A. niger*. Contaminations in Treatments 1, 4 were identified as *Rhizopus stolonifer* and formed 4.34% of total contamination. Treatments 4 and 6 had contaminants identified as *Rhizopus* sp. and formed 6.52% of total contamination. The cause of contamination in Treatment 5 (Streptomycin 300 mg dm^{-3} + Benomyl 75 mg dm^{-3}) was identified as *Cylindrocarpon* sp. and formed 4.34% of total contamination. 2.17% of total contamination formed mycelia-sterilia in Treatment 2. The rest of contaminations were unknown and they were classified under 4 types, in the same manner as in the photoperiodic condition. Colony type 1 (2.17% of total contamination, in Treatment 4), Colony type 2 (2.17% of total contamination, in Treatment 3), Colony type 3 (in Treatment 5 and the same rate contamination with Colony type 1 and 2), Colony type 4 (the cause of contamination in Treatments 2, 3, 4, 5 and 6 and formed 17.39% of total contamination) were determined.

DISCUSSION

The contamination in plant tissue cultures can originate from several sources. These can suddenly occur when there is ineffective surface sterilization and microorganisms that were concealed within explants or introduced during subculturing or via contamination which occurs simultaneously in cultures after a long period of growth (Cassells, 1990; George, 1993).

All of the types of contaminants including by fungi, bacteria, viruses, yeasts and mollicutes and rickettsias have been causing considerable economical losses in plant tissue culture laboratories (George, 1993; Leifert, 2000). For that reason it is of great importance that an effective sterilization process is developed.

The use of antibiotics for controlling plant contaminants is limited because of its impairing effect on plastids and mitochondria and chlorophyll formation (George, 1993). Reed et al. (1998) in their study phytotoxicity of antibiotics observed visually browning, chlorosis and morphological changes. In this study, phytotoxicity has not been determined by any specific phytotoxicity test.

Fungi are widespread plant pathogens and they are saprophytic soil living beings. Many fungal species have relations with plant tissues and they generally cause contamination in plant tissues. The main offenders are species of *Aspergillus, Candida, Microsporum* and *Phialophora* (George, 1993). In this study *Aspergillus* has been encountered frequently in a similar way (Table 1).

In a study concerning the effect of benomyl on shoot and root development on explants of *Asparagalus officinalis* grown in culture media, Yang (1976) reported that benomyl as a systemic fungicide had the same effect as cytokinin. Different doses (10, 25, 50, 100 and 250 mg dm^{-3}) of benomyl were added in culture medium. At low doses (10 and 50 µg g^{-1}) benomyl addition encouraged effective development, but at high levels (100 and 250 µg g^{-1}) benomyl addition affected both root and shoot formation unfavourably and it also caused an abnormally short and thick shoot development. In the present study benomyl is one of the fungicides which was used to treat *Lilium* bulb-scale explants and when used with nystatin, has been observed to be effective.

Fusarium oxysporum and *C. radicola* fungi are primary pathogens for lily. They show symptoms of illness in *Lilium* scales like tip rot and stem lesions. With the loss of bulbs in Asiatic *Lilies,* basal root illnesses are a serious problem. *F. oxysporum* together with *C. radicola* or *Phythium* caused infection in basal root. After treatment with hot water at 39°C for 2 h. Bulbs of lily, treated with in benomyl for 30 min, has caused increase in yields in *in vivo* (Lawson and Hsu, 1996). Also in our study we observed these species. In the *in vitro* culture of benomyl treatment explants, *Fusarium* had not been observed.

In order to free plant tissues from pathogens, there are some recommendations to use hot water treatments. Langens et al. (1988) determined that hot water at 40°C for *Lilium* decreased contamination, but that hot water at 45°C decreased regeneration capacity.

Kritzenger and Van Vuuren (1998), in their study about elimination of contamination in rhizomes of *Zandedeschia aethiopica*, indicated that it was possible to eliminate fungal and bacterial contaminations in rhizomes using fungicide pretreatment and antibiotic or combinations of antibiotics. According to the study results, fungicides (Captab 500 WP and Dithane M-45) showed an increasingly antibacterial effect and antibiotics (ABM1 and Imipenem) proved to be effective against contaminants.

In the culture of bulb-scale explants for *L. candidum* micropropagation, contamination has been identified and studied. After surface sterilization with Na-hypochlorite, antibiotic and fungicide combinations and treatments, it was stated that observed contamination were fungi such as *Fusarium, Penicillium, Aspergillus, Alternaria, Rhizopus* and *Cylindrocarpon* genus. In dark growth conditions, more microorganism genera were observed in *L. candidum* micropropagation cultures.

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