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Effects of Some Fungicides and Foliar Fertilizers on Epiphytic Fungal and Yeast Population of Citrus Leaves

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Abstract: In this study, the effects of some commonly used fungicides (Copper hydroxide, Diphenoconazole+Propiconazole, Iprodione, Mancozeb, Metiram and Propineb) and foliar fertilizers (Zinc, Phosphorus, Magnesium, Manganese, Potassium and Urea) were examined on phyllosphere microorganisms population on citrus leaves. As a results of the isolations from the leaves, mostly *Cryptococcus* spp. and *Sporobolomyces* spp. And *Cladosporium* spp. and *Aureobasidium* spp. were obtained in less rates. Mancozeb and Copper hydroxide had the greatest impact on mycoflora. During the period of these fungicide applications, fungal population decreased by approximately 50-fold. Effects of these fungicides continued up to 6 months after application. Diphenoconazole+Propiconazole and Iprodione had no significant effect on the fungal population. Effect of Metiram and Propineb fungicides disappeared after 2 months of applications. Although foliar fertilizers seemed to have a negative impact on fungal populations, repeated applications increased the mycoflora. Especially foliar fertilizers such as zinc and phosphorus remarkably increased *Sporobolomyces* species.

Bazı Fungisit ve Yaprak Gübrelerinin Turunçgil Yapraklarındaki Epifitik Fungus ve Maya Populasyonları Üzerine Etkileri

Anahtar Kelimeler
Fillosfer mikoflorası,
Mayalar,
Fungisit,
Yaprak gübresi

Özet: Bu çalışmada, turunçgillerde yaygın kullanımı olan bazı fungisit (Bakır hidroksit, Diphenoconazole+Propiconazole, Iprodione, Mancozeb, Metiram ve Propineb) ve yaprak gübrelerinin (Çinko, Fosfor, Magnezyum, Mangan, Potasyum ve Üre) fillosfer mikoflorası üzerine etkileri araştırılmıştır. Yapılan izolasyonlarda, çoğunlukla *Cryptococcus* spp. (Beyaz maya) ve *Sporobolomyces* spp. (Pembe maya), daha az oranda ise *Cladosporium* spp. ve *Aureobasidium* spp. elde edilmiştir. Mancozeb ve Bakır hidroksit, mikoflorayı en fazla etkileyen fungisitler olmuştur. Bunların uygulandığı dönemde fungal populasyon 50 kat civarında azalmıştır. Bu fungisitlerin etkisi, uygulandıktan sonra 6 ay devam etmiştir. Fungal populasyon üzerine Iprodione ve Diphenoconazole+Propiconazole'un önemli bir etkisi olmamıştır. Metiram ve Propineb'in etkileri ise uygulama kesildikten 2 ay sonra ortadan kalkmıştır. Yaprak gübreleri, başlangıçta fungal populasyonu olumsuz etkiliyor gibi görülse de tekrarlanan uygulamalarda mikofloranın artışına katkı sağlamıştır. Özellikle Çinko ve Fosfor içeren yaprak gübreleri *Sporobolomyces* spp.'nin populasyonunu önemli düzeyde arttırmıştır.

1. Introduction

Leaf surface called as phyllosphere is inhabited by many microorganisms. The global surface area of the phyllosphere has been estimated to over 4×10^8 km^2. The large number of bacteria on leaves in temperate regions of the world and populations in tropical regions are probably even larger, the planetary phyllosphere bacterial population may be as large as 10^{26} cells [1]. The microbial communities of the phyllosphere are divers, supporting numerous genera of bacteria, flamentous fungi, yeasts, algae and in some situations protozoans and nematodes [2,3,4]. A study on the estimation of the diversity of phyllosphere bacteria in the 20.000 vascular plants inhabiting the Brazilian Atlantic forest, suggested the possible occurrence of 2-13 million phyllosphere bacrerial species in this habitat alone [5]. Many of these species are gram-negative and belong to genera *Erwinia*, *Pseudomonas*, *Flavobacterium* and

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Xanthomonas [6]. The most common groups of fungi are yeasts; *Cladosporium*, *Aureobasidium*, *Alternaria* and *Epicoccum* species. White yeasts (*Cryptococcus* spp.) and Pink yeasts (*Sporobolomyces* spp.) are the dominant species [7].

Epiphytic filamentous fungi, yeasts and bacteria may arrive on the leaf surface though insect, atmosphere, seed and animal borne sources [8]. Microorganisms may occur individually on the leaf surface but frequently, they occur as aggregates or biofilm like structures containing bacteria [9].

Composition and concentration of atmospheric microflora, may vary daily and seasonal as a result of environmental events such as rain and strong wind that directly affect the transport from phyllosphere [10].There are seasonal effects on the microorganisms on leaves. The numbers of all microorganisms are low at the beginning of leaf development. The population soon increases however as the bud flora multiplies and new inoculums arrieves from the surrounding air. Populations usually reach a peak in autumn as the leaves senesce [11].

Phyllosphere microorganisms can be affected by the other organisms come to leaf surface. Stadler and Muller [12] reported the impact of aphid infestation on the phyllosphere microflora of *Picea abies*. The development of fungi and yeast were significantly increased with the release of honeydew as a result of feeding aphids. Honeydew is reported as a potential energy source that supports the growth of microorganisms. Moreover pollen and substances such as plant and insect exudates on the leaf surface are also food source for these microorganisms.

In addition, suitable moisture and the absence of free water on the leaf surface are also limiting factors for the colonization of microorganisms. In a study, it has been found that spores of *Alternaria* and *Cladosporium* spp. developed at 97% humidity whereas spores of *Sporobolomyces roseus* developed at 85% humidity [13]. Another study is related with negative impact factors on phylosphere microorganism populations showed that UV light was one of the most effective factor on these microorganisms [14,15] .

Microbial interactions in the phyllosphere can affect the fitness of plants in natural communities, the productivity of agricultural crops and the safety of horticultural produce for human consumption [16]. These microorganisms can compete with pathogens for nutrients, inhibit development of pathogens by producing volatile or nonvolatile antibiotics or promoting plant for phytoalexin synthesis. Furthermore phyllosphere microorganisms may also show parasitic effect on pathogens.

These microorganisms which have important functions for plant health, should be included in integrated disease management programs. Furthermore, these microorganisms which have antagonistic effects on plant pathogens in the same ecological niche can be used as biological control agents. In a study, antagonist fungi were not able to inhibit *Phoma tracheiphila*, a causal agent of Citrus Mal Secco disease, infections on young lemon trees, but reduced the disease severity and infection rate of twigs [17].

It should be considered that chemical applications cause harmful effect not only pathogens but also non-target microorganisms such as antagonistic bacteria and fungi on leaf surface microflora. Today, intensive chemical sprays change the natural balance of the phyllosphere microflora in favors of pathogens. For instance, eight or ten different fungicides can be applied only in a season in Adana, eastern Mediterranean region of Turkey. After the applications, number of microorganism species decline, composition and diversity of the microorganisms change, and some microorganisms even disappear from the phyllosphere microflora. Finally, the natural balance on the leaf surface may be changed by intensive fungiside applications.
In this study, effects of some commonly fungicides and foliar fertilizers were examined on the phylloshere fungi and yeast microorganisms. The population dynamics of these microorganisms were observed according to seasonal and climatic changes.

2. Material and Method

2.1. Chemical applications

This study was conducted on eight years old mandarin (cv. Hernandina) trees in citrus orchards where common fungicides and foliar fertilizer used (Table 1 and Table 2). Fungicides and foliar fertilizers were applied with back sack sprayer at the recommended dosage.

Table 1. The fungicides used in the experiment

Active ingredients	Trade name	Formulation	Manufacturing company	Recommended dosage (100 L water)
Copper Hydroxide	Kocide	DF	Du Pont	300 g
Diphenoconazole+ Propiconazole	Harbor	EC	Safa	50 ml
Iprodione	Koruval	WP	Koruma	100 g
Mancozeb	Dikotan M-45	WP	Koruma	400 g
Metiram	Polyram	DF	BASF	200 g
Propineb	Antracol	WP	Bayer	250 g

Table 2. The foliar fertilizers used in the experiment

Active ingredients	Trade name	Formulation	Manufacturing company	Recommended dosage (100 L water)
Zinc	Grow Zn-10	EC	Swiss-Grow	200 ml
Phosphorus	Magnum	EC	Nutri Phite	200 ml
Magnesium	Magnisal	WG	Toros	500 g
Manganese	Grow Mn-10	EC	Swiss- Grow	200 ml
Potassium	Potasyum Nitrat	WG	Toros	2000 g
Urea	Urea Low Biuret	WG	Bİolchim	500 g

2.2. Determination of the effects of fungicides and foliar fertilizer on annual population growth of phyllosphere mycoflora

In the study, the first chemical application was taken place onto the one month old, first spring shoots. Fungicides and foliar fertilizers applied 6 times in 6 months period every 30 days. Plant samples were taken 15 days following applications for microbial isolation. Fungicides and foliar fertilizer application were stopped after 6 months. Thereafter, isolation has been continued once a month to determine long term effects of chemicals until population fluctuation back to normal. For all isolation, leaves from the spring shoot were used.

2.3. Isolation and identification of phyllosphere mycoflora

To isolate phyllosphere mycoflora from each leaf samples brought to laboratory, 1 cm^2 pieces were cut and put into 250 ml Erlenmayer containing with 100 ml sterilized distilled water, shaked at 180 rpm for 30 minutes. This suspension diluted down to 10^{-2} and from the series of the diluted solution 0,1 ml spread onto Potato Dextrose Agar (PDA) media [16]. Petri plates were incubated at 24^0C for five days. Growing colonies after incubation were examined macroscopic and microscopic level and the fungal populations of 1cm^2 leaf area was calculated as propagules. After incubation different colonies were identified at the genus level. For identification, filamentous fungi were examined colony colour, sexual and asexual spore structures, properties such as shape conidiophores and conidia. In addition yeast were identified at the genus level according to criteria such as colony color, sexual spore formation, cell colour, shape and size examined under microscope [18].

3. Results

3.1. Population development of phyllosphere mycoflora

In Hernandina Mandarin orchard, results of population throughout the year revealed that phyllosphere mycoflora contains mainly *Cryptococcus* and *Sporobolomyces* species (Table 3). *Cladosporium* and *Aureobasidium* species which were not reach high population levels, also isolated. Under the high humidity and temperature conditions, *Cladosporium* species showed a slight increase and reached 10.200 propagules in September (Figure 1). Also *Aureobasidium* species, have a small amount of a population on the young leaves in spring (3200 propagules in May), later on the amount of population gradually decreased. Furthermore, have also been isolated at low rates of *Aspergillus*, *Penicillium*, *Alternaria*, *Epicoccum* and *Fusarium* species, from phyllosphere.

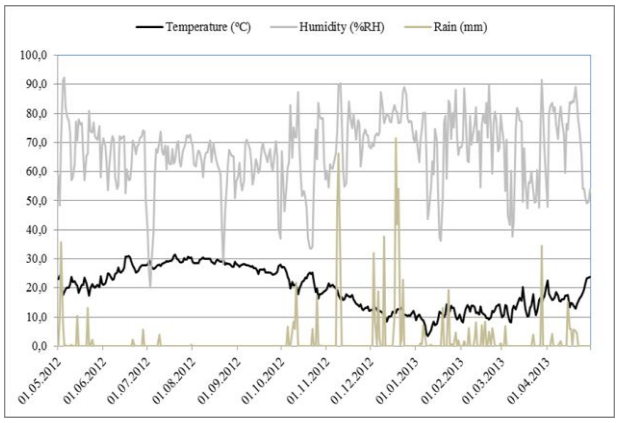

Figure 1. Annual climate data of experimental area

Widespread phyllosphere fungi isolated throughout the year populations vary according to the microorganism species, leaves size and seasonal events. The amount of total fungi on leaf surface, at the first isolation in May, due to fresh leaves at the beginning of seasons, measured at low level about 22.4 x 10^3 propagules /cm^2 leaf, while it have gradually increased until September. In August, under the highest temperature and relative humidity conditions, the total amount of fungi reached to the highest level (85 x 10^3 propagules / cm^2 leaf). Starting from August, parallel to temperature decrease and due to rainy period, the epiphytic microflora also decrease remarkably. In three months period between September to November, the fungal microorganism population has decreased gradually due to scattered rains and decreasing temperatures. In December, more long-term high continues rainfall and low temperatures, epiphytic microorganism population on leaves recorded the lowest level with 5 x 10^3 propagules. In January increasing in temperatures and decrease in rainfall which has prevented washing of leaf microflora have positive effect on the development of the mycoflora population. The leaf of the previous year's spring shoots, when they mature enough, was a determining factor in achieving high population levels. In April, when the last isolation was made the total amount of microorganisms was measured in 1 cm^2 leaf area over 120.000 propagules.

Table 3. Annual population development of phyllosphere mycoflora (Propagules * 10^3 / cm^2 leaf)

	Aureobasidium spp.	*Cladosporium* spp.	*Cryptococcus* spp.	*Sporobolomyces* spp.	Other fungi	Total fungi
May	3,1 a	3,6 c	11,4 ef	3,7 ef	0,1 d	22,4 ef
June	1,8 b	1,6 d-f	33,7 cd	5,7 d-f	0,3 d	43,3 c-e
Jully	0,7 cd	5,2 b	38,9 c	11,5 c-f	0,6 cd	56,8 c
August	0,3 de	3,6 c	54,9 b	27,9 ab	1,2 b-d	85,0 b
September	0,9 c	10,2 a	18,6 d-f	18,8 bc	2,3 b	49,1 cd
October	0,0 e	2,9 cd	16,1 ef	19,2 bc	2,0 bc	39,9 c-e
November	0,0 e	2,3 cd	30,3 c-e	15,9 cd	7,2 a	54,7 cd
December	0,0 e	0,2 f	2,9 f	1,5 f	0,4 cd	5,0 f
January	0,1 e	0,4 f	9,4 ef	13,9 c-e	7,2 a	28,3 d-f
February	0,0 e	0,8 ef	23,3 c-e	14,1 c-e	2,1 bc	40,3 c-e
March	0,0 e	2,2 c-e	83,6 a	32,9 a	1,1 b-d	120,5 a
April	0,0 e	1,6 d-f	58,5 b	36,3 a	8,1 a	126,8 a

3.2. Effects of fungicide application on phyllosphere mycoflora

In this study, the toxic effects of fungicides on the saprophytic fungi on leaf surface that applied onto the vegetative parts of the plants, was evident. Reducing effect of Iprodione application on phyllosphere populations have not significant (Table 4.). Similarly, Diphenoconazole + Propiconazole application have not been a significant effect. Copper hydroxide, Mancozeb, Propineb and Metiram fungicides applications severely suppressed mycofloral population in all months and has affected the microorganism population at rates ranging between 93-96% in October, after the last application. Since November, microorganism populations began to increase due to Propineb and Metiram applications, but reducing effect of Mancozeb and Copper hydroxide applications continued until February. Mancozeb and copper hydroxide have the greatest impact on microfloral fungal population. Fungal population decreased about 50 fold during the application period. The negative effects of these fungicides, was continued for 6 months following application. Although Mancozeb application had highly toxic effect on pink and white yeast, application of this fungicides encouraged the development of *Cladosporium* species. On the other hand both fungicide applications, have not a significant effect on *Aureobasidium* population.

3.3. Effects of foliar fertilizer application on the phyllosphere mycoflora

Initially foliar fertilizers, caused negative impact on fungal population, but later increase in the microflora was determined with repeated application (Table 5.). In this study, fungal population was not found at very low levels until the last application in October. From October, the phyllosphere mycoflora population gradually increased then population were measured more than control in all fertilizer application after December. Foliar fertilizers, have an increasing effects on the microorganism population at rates changing 54-210% in January. Pink and white yeast species encouraged similarly from the foliar fertilizer application and reached its highest population level after end of applications. Particularly, zinc and phosphorus containing foliar fertilizers, increased the *Sporobolomyces* spp. population significantly. On the other hand, *Cladosporium* and *Aureobasidium* species were not affected by these applications.

4. Discussion and Conclusion

According to result of isolations phyllosphere mycoflora of treated leaves of Hernandina mandarin leaves revealed that *Cryptococcus* and *Sporobolomyces* species were the most common fungal species. In addition *Cladosporium* and *Aureobasidium* species were also isolated rarely through out a year. Campbell [11], has been reported that *Aureobasidium pullulans* becomes dormant by producing chlamidospore after summer months, but routinely isolated in small quantities. Furthermore, *Aspergillus*, *Penicillium*, *Alternaria*, *Epicoccum* and *Fusarium* species, from phyllosphere of mandarin leaves have been also isolated at low rates. The population fluctuations of these fungus change. Treatments according to leaf age, season and climatic conditions. Sadasivam et al [20], have been reported that bacteria and fungi on phyllosphere increases with the aging of the leaves. Fungicide applications caused negative effect on fungal populations on the leaf surface. Both Mancozeb and Copper hydroxide were the most effective fungicides on mycoflora. The negative effects of these fungicides last long 6 months. Iprodione and Diphenoconazole+Propiconazole were not shown to possess severe effects on fungal populations. The negative effects of Propineb and Metiram disappeared 2 months after application, then the fungal population started to increase again.

At the beginning foliar fertilizers seemed to they have negative effect following repeated applications. They caused mycoflora populations to increase. Especially foliar fertilizers such as zinc and phosphorus, had increasing effect on *Sporobolomyces* spp. population remarkably.

Table 4. Effect of fungicide application on total fungal population on the leaves (prop. * 10^3 / cm^2 leaf)

Treatments	May	June	Jully	August	September	October
CuOH	6,0 a-c	2,3 a	3,3 ab	2,0 ab	3,1 ab	2,5 a
Iprodione	26,0 d	37,3 c	48,1cd	157,3 d	55,9 c	32,3 b
Mancozeb	1,6 a	1,3 a	2,1 a	1,3 a	6,3 b	3,0 a
Metiram	18,5 b-d	2,3 a	11,7 bc	2,8 b	2,4 ab	2,3 a
Diphenoconazole+Propiconazole	13,9 b-d	12,5 b	56,0 d	11,5 c	47,1 c	24,1 b
Propineb	3,8 ab	1,9 a	2,4 a	1,4 ab	1,5 a	1,5 a
Control	22,4 cd	43,3 c	56,8 d	85,0 d	49,1 c	39,9 c
Treatments	November	December	January	February	March	April
CuOH	2,8 a	5,9 b	5,0 a	10,3 a	64,8 ab	98,0 a
Iprodione	78,0 d	8,6 b	74,1 d	62,1 c	151,3 c	148,8 a
Mancozeb	4,2 ab	0,2 a	8,5 ab	16,3 ab	26,4 a	91,6 a
Metiram	12,9 c	0,7 ab	59,9 cd	27,6 bc	103,0 bc	106,0 a
Diphenoconazole+Propiconazole	56,9 d	2,7 b	51,1 cd	43,8 bc	168,7 c	100,3 a
Propineb	10,1 bc	1,3 b	18,8 bc	27,8 bc	207,2 c	157,3 a
Control	54,7 d	5,0 b	28,3 cd	40,3 bc	120,5 bc	126,8 a

Table 5. Effect of foliar fertilizer application on total fungal population on the leaves (prop. * 10^3 / cm^2 leaf)

Treatments	May	June	Jully	August	September	October
Zinc	6,9 a	15,5a	27,3 ab	18,6 a	24,8 a	20,0 a
Phosphorus	14,6 ab	61,1 a	111,1 d	61,3 bc	65,7 a	75,7 c
Magnesium	27,2 b	33,0 a	56,1 cd	48,7 bc	42,2 a	26,9 ab
Manganese	12,1 ab	47,7 a	39,5 bc	44,9 b	26,6 a	47,0 bc
Potassium	7,4 a	51,4 a	46,5 bc	24,4 ab	28,1 a	38,2 b
Urea	10,4 ab	25,4 a	18,8 a	57,2 bc	31,2 a	15,2 a
Control	22,4 b	43,3 a	56,8 cd	85,0 c	49,1 a	39,9 b
Treatments	November	December	January	February	March	April
Zinc	104,2 b	7,4 a	87,4 c	66,3 a	248,1 a	274,4 a
Phosphorus	97,6 b	4,3 a	75,1 bc	91,7 a	169,4 a	186,4 a
Magnesium	49,8 a	6,0 a	48,4 bc	65,5 a	139,1 a	177,8 a
Manganese	48,3 a	3,6 a	43,5 ab	46,2 a	135,6 a	160,3 a
Potassium	43,3 a	2,6 a	48,9 bc	53,2 a	120,2 a	161,4 a
Urea	56,9 ab	3,6 a	52,2 bc	55,4 a	147,9 a	182,3 a
Control	54,7 a	5,0 a	28,2 a	40,3 a	120,5 a	126,8 a

In this study the fungicides, which were used to control plant pathogens, had an effect onto environment by inhibiting beneficial mycoflora on the leaf surface. Unnecessary use of chemicals should be avoided due to this effect.

The results of the recent studies demonstrate that commonly used fungicides had moderate but significant effect on fungal community composition in the wheat phyllosphere [21]. Similarly in another study, application of Imidacloprid and Metalaxyl pesticides induced mild effects on the fungal and bacteriel communities of pepper plant. The only exception was the foliage application of Imıdacloprid which showed a more prominent effect on the fungal community[22].

Foliar fertilizers application could also encourage saprophytic mycoflora on leaf surface however phytotoxicity probability should be keep in mind by the repeated applications. However in a recent study, mineral fertilization reduced the total number of fungi colonizing the phyllosphere of fodder galega[23].

The negative effects of fungicides on phyllosphere mycoflora may be decrease by using these chemicals mixed with foliar fertilizers.

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