Effect of foliar nutrients, fungicides, temperature and metal ions on pectate lyase and endopolygalacturonase from *Alternaria alternata* found in association with sooty molds on Citrus trees.

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ABSTRACT

The properties of endopolygalacturonase and pectate lyase, produced by *Alternaria alternata* isolated from sooty mold infected *Citrus* trees were studied. These enzymes, related to plant pathogenesis, were obtained from supernatants from liquid Mandels medium inoculated with fungal conidia which contained Citrus pectin as the sole carbon source. The behavior of the enzymes towards metal ions, foliar fertilizer and fungicides, was analyzed. Also, the effect of temperature was tested. Results show that at the suggested concentrations to be used at the field level for foliar nutrients and fungicides, these enzymes maintain enough activity to produce damage on the plant tissues. Also, the high levels of enzyme activities found in the presence of high concentrations of metal ions or at different temperatures could relate to the behavior of these enzymes in the infection process of plant tissues in field conditions. FITOPATOLOGIA 28(1) 1993:38-44.

INTRODUCTION

One of the mechanisms that plant pathogens can use to infect plant tissues is the secretion of pectin degrading enzymes such as endopolygalacturonase (EPGase) and pectate lyases (PLase), to initiate the degradation of the plant cell wall (1, 5, 7, 11). Most of phytopathogenic fungi can produce these enzymes (3, 8), although some bacteria have been described as pectinase producing species, specifically those corresponding to the genus Erwinia (1, 5). Some work has been done related to the inducers of the production of EPGase and PLase, as well as the identification of genes codifying for these enzyme activities (5). EPGase has been considered as an elicitor of the plant cell response (13), and the effect of calcium on enzyme activity has been described for several plant systems (4, 6, 14). Nevertheless, little work has been done to look at enzyme activity in experiments that could mimic field conditions. *Alternaria alternata* and several other fungal species have been isolated from sooty mold fungi that infect Citrus trees; and their ability to produce both endopolygalacturonase and cellulase has been tested (17). Alternaria diseases are among the most common diseases of many plants throughout the world (1). They affect primarily the leaves, stems, flowers and fruits of annual plants, specially vegetables and ornamentals, but also may affect these same parts in trees such as *Citrus* and apples among others (12). The present work describes the effect of foliar fertilizers, fungicides, temperature and metal ions on the activities of EGPase and PLase produced by *Alternaria alternata* assayed under conditions that could occur at the field level.

MATERIALS AND METHODS

Inducing media

Alternaria alternata isolated from sooty mold (17) was grown on Mandels mineral salts medium (15) with the addition of 1.8 g l^{-1} agar DIFCO and 4.0 g l^{-1} *Citrus* pectin (Sigma) as sole carbon source. Plates were incubated at 28° C for 5 to 7 days.

Production of extracellular enzymes in shake flasks.

Submerged cultures were prepared in Mandels mineral salts medium (15) with the addition of 4.0 g l^{-1} *Citrus* pectin (Sigma). Flasks containing 200 ml

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Aceptado para publicar: Marzo 17, 1993.

were inoculated with a total of 8×10^6 conidia, collected from the inducer medium according to the method by AOAC (2) and were incubated at 28° C for 4 days in an orbital shaker incubator (New Brunswick) at 150 rpm. The whole medium was centrifuged at 9,000 x g for 10 min to remove solids, and the supernatant was analyzed for enzymatic activity.

Enzymatic determinations.

Endopolygalacturonase.

EPGase activity was assayed in 50 mM sodium acetate pH 4.8 containing 5 g l^{-1} sodium polygalacturonate (Sigma). Reaction mixtures were incubated 1 hour at 37° C, and formation of reducing ends was measured by the Nelson-Somogyi method (16, 18). Enzyme activity was expressed as a percentage, considering as 100% activity the one obtained in the above assay conditions.

Pectate lyase.

PLase activity was assayed in 50 mM TRIS-HCl pH 8.5, containing 5 g l^{-1} sodium polygalacturonate and 1 mM $CaCl_2$. Reaction mixtures were incubated up to 45 min at 37° C and formation of the double bond was monitored at 235 nm, as described (8). Activity was expressed as change 235 nm per minute and per ml of supernatant.

Controls.

Non enzymatic controls were prepared with boiled supernatant (40 min), and were subtracted from the corresponding enzymatic values.

Effect of metal ions.

The effect of different concentrations of Cu^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, as the corresponding chlorides (Merck), was tested on EPGase and PLase activities. An aliquot of the corresponding metal ion was added to the assay medium, and enzyme activities were measured as above.

Effect of foliage nutrient.

The effect of different concentrations of a liquid foliar nutrient (Bayfolan) was tested on EPGase and pectate lyase activities. An aliquot of the proper dilution of the foliar nutrient was added to the assay medium, and enzyme activities were measured as above.

Effect of fungicides.

Aliquots of different dilutions of fungicides (Captan, Benomyl) were added to the assay medium, and EPGase and PLase activities were measured as above.

Effect of freezing-thawing.

Several cycles of freezing-thawing of the enzymes were performed before measuring EPGase and PLase activities as above.

Effect of temperatures.

Preincubations of the enzymes at different temperatures, during different time periods were performed before measuring EPGase and PLase activities, as mentioned above.

RESULTS AND DISCUSSION

Effect of metal ions.

Endopolygalacturonase activity.

The effect of the different metal ions on the enzyme activity is shown in Table 1, along with the concentrations of the same metal ions that are commonly found in foliar nutrients and in fungicides. The enzyme activity in the presence of Ca^{2+}, Cu^{2+} and Zn^{2+} slightly increased in the range of concentrations from 0 to 100 µM, and then decreased to an activity similar to the one obtained in the absence of added metal ion. With Mn^{2+} there was no change in activity up to 1,000 µM. In the presence of Fe^{2+}, Fe^{3+} and Co^{2+} there was a decrease at all concentrations tested. It is noteworthy to mention that metal ion concentration in foliar nutrients ranges among 2 and 9 µM. Ca^{2+} is not a normal component of foliar nutrients. Cu^{2+} is normally found at concentrations of 3 - 8 µM, which did not inhibit EGPase but produced an small increase in its activity (10-20%). Concentrations of 2 - 5 µM Zn^{2+}, which is normally found in foliar nutrients, maintained 100% activity of EPGase. Concentrations of 5 - 9 µM of Fe^{2+}, Fe^{3+} and Mn^{2+} are found in foliar nutrients. While Mn^{2+} did not affect EGPase activity, both Fe^{2+} and Fe^{3+} inhibited 4 and 17% of basal EGPase activity. Co^{2+} did not alter enzyme activity when tested at concentrations of 3 - 5 µM, which are normally found in foliar nutrients. If all these metals ions are together in a foliar nutrient, the total ion concentration will be 23 - 45 µM, concentration that did not affect enzyme activity. These results suggest that EPGase is slightly sensitive to the presence of metal ions, and therefore high concen-

Table 1. Effect of different metal ions on the activity of endopolygalacturonase

	% Activity[a]						
	Cu^{2+}	Cu^{2+}	Fe^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}	Ca^{2+}
Ion Con. (μM)							
0[b]	100	100	100	100	100	100	100
2.5	114	96	94	100	100	99	n.d.
5	116	92	88	98	99	100	n.d.
10	121	96	83	101	99	118	n.d.
50	125	96	89	100	101	119	n.d.
100	130	92	89	100	104	120	112
500	110	93	89	102	98	119	n.d.
1,000	102	94	75	100	49	111	n.d.
10,000	95	58	75	65	28	101	93
Ion conc. in foliar fertilizer (μM)[c]	3-8	5-9	5-9	5-9	3-5	2-5	
Ion conc. in fungicides (mM)[d]	20	—	—	2.2-4.7	0	—	—

a Results correspond to the mean of three experiments with duplicates.
SD did not exceed 10%. 1mM EDTA was added to the assay media.
n.d. not determined.
c Ref. (20). Ref(10)

trations of them must be used to inhibit the activity. Concentrations of 20 mM Cu^{2+} and 2.2 Mn^{2+} are used in fungicides. EPGase is 5% inhibited by 10 mM Cu^{2+} and a higher inhibition could be expected at 20 mM of the ion. At this same concentration (10 mM), EGPase is only 35% inhibited by Mn^{2+}, and therefore at the concentration in the fungicide, enzyme activity would be higher than 65%.

On the other hand, the reported concentration of some of these metal ions inside plant tissues is high enough to inhibit EGPase activity. Nevertheless, it should be considered that most of these metal ions are not in a free form but associated to proteins and other cellular structures (19) and, if they were in a free form they could inhibit EPGase only if the enzyme would penetrate inside the plant tissue. Therefore, it could not be expected that cellular metal ions could inhibit the EPGase. If this type of enzyme is secreted by a pathogenic fungus, it will retain its full activity if plant surface or plant inner concentrations of the metal ion are lower than 100 μM. The inhibition of EGPase activity by increasing concentrations of Ca^{2+} ion has been reported (14), as well as the resistance to infection of plant tissues with high Ca^{2+} content (4). Nevertheless, Ca^{2+} concentrations used by McGuire and Kelman (14) for the inoculation of apple fruits, reached 0,36 M; which is much higher than the concentrations used in the present work.

Pectate lyase activity.

The effect of the different metal ions on PLase activity is shown in Table 2. Unlike EPGase, PLase requires metal ions for its activity. The presence of 1 mM EDTA abolished completely enzyme activity. All the metal ions tested increased PLase activity. The highest activity was observed when Ca^{2+} was the cofactor, but all the other metal ions could replace Ca^{2+}, although less efficiently. These results indicate that even if Ca^{2+} is at limiting low concentrations, the enzyme will have enough activity in order to dissolve pectin components of the plant cell wall. Concentrations higher than 1 mM could not be assayed because of their precipitation at the pH of the assay medium, forming a complex with polygalacturonic acid. The effect of most metal ions, with the exception of Zn^{2+}, showed two peaks of activity at 10 μM and 500 μM, suggesting that there could be present two PLases with a different optimal ion concentration requirement for full activity. Another possibility could include the presence of a special type of complex between PGA and the metal ion which could provide the proper conformation for substrate hydrolysis. It could be expected from the results of Table 2, that higher concentrations of all metal ions would inhibit PLase activity.

Low concentrations of these metal ions will have a severe effect on plant nutrition (1), even though these conditions will decrease enzyme activity. Therefore, at good nutritional conditions of the plant, PLase activity will not be affected, thus allowing it to produce damage on the plant surface. On the other hand, the use of foliar nutrients will enhance PLase activity. Therefore, it could be expected that plants that have been treated with foliar nutrients will be more sensitive to damage by PLase than plants not treated, because of the enhancement of the PLase activity in the presence of metal ions.

The dependence of Ca^{2+} for enzyme activity has been described for other PLases (8). Table 2 shows that pectate lyase activity in the presence of all Ca^{2+} concentrations did not change abruptly as compared to the effect of the other metal ions tested. It has been reported that Ca^{2+} extracellular concentration reaches 1,000 µM in pea root tissue (19); a concentration that could be found in most plant tissues. 500-1,000 µM Ca is enough to obtain full activity of PLase. Cellular metal ion concentrations that inhibit EPGase activity would stimulate PLase.

Effect of foliar nutrients.

The effect of different dilutions of foliar nutrient on EPGase and PLase is shown in Table 3. PLase resulted more susceptible to the foliar nutrient than EPGase. At dilutions lower than 1 : 80 no PLase activity could be detected. On the other hand, EPGase maintained almost full activity at dilutions of 1 : 160 of the foliar nutrient. EPGase retained 90% of activity at dilutions of the foliar nutrient recommended for use at the field level (1 : 200 dilution). Under the same conditions, PLase retained only approximately 54% activity. These results show that the foliar nutrient tested did not inhibit EPGase and PLase activities completely, thus allowing the enzymes to dissolve pectin components of the plant cell wall. It is important to notice that although both enzymes retain a high activity in the presence of high concentrations of individual metal ions, they are inhibited by the foliar nutrient which contains a mixture of them.

Effect of fungicides.

The effect of different concentrations of fungicides on EPGase and PLase is shown in Table 4. EPGase increased its activity at all Captan concentrations tested. A small increase was observed at low concentrations of Benomyl with a further inhibition at high concentration. Conversely, PLase decreased its activity at all Captan and Benomyl concentrations used. Both Captan (surface fungicide) and Benomyl (systemic fungicide) had a similar effect on PLase. At the concentrations used at the field level (1% w/v for Captan and 0.3% w/v for Benomyl), complete inhibition of PLase activity would be expected. On the other hand, EPGase would be expected to maintain full activity with 1% w/v Captan and 60% activity in the presence of 0.3% Benomyl.

The direct effect of both fungicides on fungal growth has been reported (12). However, if the fungus has

Table 2. Effect of different metal ions on the activity of pectate lyase

	Activity (O.D. 235nm ml^{-1} min^{-1} x 10^6)[a]				
	Cu^{2+}	Cu^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}
Ion Con. (µM)					
0[b]	0	0	0	0	0
5	4,276	1,840	3,714	52	3,500
10	4,631	2,717	4,646	422	4,100
50	4,307	2,102	3,914	20	3,400
100	4,441	4,710	6,740	2,516	2,800
500	5,740	5,979	10,508	6,284	3,200
1,000	5,516	0	8,001	3,096	5,700

a Results correspond to the mean of three experiments with duplicates.
SD did not exceed 10%. Due to an undetectable enzyme activity in the absence of added metal ion, results are reported as mentioned and not as a percentage.
b 1mM EDTA was added to the assay media.

Table 3. Effect of foliar nutrient on endopolygalacturonase and pectate lyase activities

	% Activity[a]	
Foliar nutrient (dilution)	Endopoly-galacturonase	Pectate lyase[b]
0	100	100
1 : 640	98	91
1 : 320	96	54
1 : 160	90	10
1 : 80	88	0
1 : 40	85	0
1 : 20	47	0
1 : 10	39	0
1 : 5	6	0

a Results correspond to the mean of three experiments with duplicates.
SD did not exceed 10%.
b Activity was measured in the presence of 0.5 mM Ca^{2+}.

previously produced EPGase, this enzyme could produce plant damage because of the inability of the fungicides to inhibit this enzyme.

Effect of freezing-thawing.

The effect of several cycles of freezing-thawing of both EGPase and PLase was tested. The occurrence of freezing conditions at the field level is common in winter time in several countries. These conditions could affect plant structures, producing damage and tissue destruction. This could facilitate fungal infections and enhancement of fungal enzyme activities when weather conditions improve. Therefore, it is important to test whether these enzymes retain their activity after freezing and thawing. After five cycles, both enzymes maintained full activity. These results suggest that freezing conditions at the field level will not affect enzyme activity. Moreover, both enzymes could be stored for more than one year at -20°C retaining full activity.

Effect of temperature.

Temperatures change during day and night times, and these changes could affect enzyme activities. The effect of preincubation at different temperatures for different times on the EGPase activity is shown in Table 5. Preincubation at 100°C during 5 minutes showed a 67% decrease in EPGase activity, and it continued to decrease when the preincubation time was enlarged. Heating at 100°C for 40 minutes ensured that EPGase was completely inactivated. A similar behavior was observed for PLase (Table 6), but its thermal stability is lower than the observed for EPGase. Although these thermal conditions are never produced at the field level, this demonstrates the unusual stability to high temperatures of both EGPase and PLase. Also, both activities remained unchanged when they were maintained at room temperature for at least 48 hours, time enough to produce damage on the plant surface.

The overall effects observed when testing the EPGase and PLase activities show that these enzymes have great stability. This means that these enzymes could damage the leaf surface under conditions where the fungus that produce them could not survive, as has been described for the direct treatment of Citrus tissues with the same source of enzyme used in this work(9).

Table 4. Effect of fungicides on endopolygalacturonase and pectate lyase activities

Fungicide (% w/v)	% Activity[a]	
A) Captan	Endopoly-galacturonase	Pectate lyase[b]
0	100	100
0.075	110	96
0.15	115	77
0.30	115	16
0.45	135	n.d.
0.60	n.d.	0
0.75	135	0
1	n.d.	0
1.5	n.d.	0
B) Benomyl		
0	100	100
0.05	110	22
0.1	83	10
0.2	70	1
0.4	47	0
0.8	34	0

a Results correspond to the mean of three experiments with duplicates.
SD did not exceed 10%.
b Activity was measured in the presence of 0.5 mM Ca^{2+}.

Table 5. Effect of preincubation at different temperatures on endopolygalacturonase activity

Time (min)	% Activity				
	0° C	4°C	24°C	50°C	100°C
0	100	100	100	100	100
5	100	100	100	157	33
10	100	100	100	131	27
20	100	100	100	121	4
40	100	100	100	111	0
60	100	100	100	98	0

ACKNOWLEDGEMENTS

This work was funded by grants 157/88 and 91/0886 from FONDECYT.

Table 6. Effect of preincubation at different temperatures on pectate lyase activity

Time (min)	% Activity				
	0° C	4°C	37°C	60°C	100°C
0	100	100	100	100	100
2	100	100	100	100	89
5	100	100	98	102	57
10	100	100	100	89	13
20	100	100	97	62	0
40	100	100	90	41	0
60	100	100	86	17	0

RESUMEN

EFECTO DE NUTRIENTES FOLIARES, FUNGICIDAS, TEMPERATURA Y IONES METÁLICOS EN PECTATO LIASA Y ENDOPOLIGALACTURONASA DE *Alternaria alternata* AISLADA DE PLANTAS DE CITRICOS ATACADAS DE FUMAGINA. M. Aubá, M. Chiong y L. M. Pérez . Departamento de Bioquimica y Biologia Molecular, Facultad de Ciencias Quimicas y Farmacéuticas, Universidad de Chile. Casilla 233 Santiago 1, CHILE.

Se estudian las propiedades de las actividades de pectato liasa y de endopoligalacturonasa, enzimas relacionadas con la patogenicidad de hongos, producidas por *Alternaria alternata*, hongo que se ha encontrado asociado con la fumagina que infecta citricos en Chile. Las actividades enzimáticas se obtuvieron de sobrenadantes de Medio de Mandels inoculados con *A. alternata* y cuya única fuente de carbono era pectina citrica. Se analizó el comportamiento de ambas enzimas frente a diferentes metales bivalentes, frente a nutrientes foliares y frente a fungicidas. Se analizaron además, las propiedades de termoestabilidad de ambas enzimas. Los resultados muestran que a las concentraciones de nutrientes foliares y fungicidas sugeridas para su uso a nivel de campo, estas enzimas mantienen suficiente actividad para producir un daño en el tejido vegetal. Además, el mantenimiento de su actividad frente a altas concentraciones de iones metálicos y cambios de temperatura, permiten explicar porqué estas enzimas ven favorecida su actividad colaborando al proceso infectivo de hongos patógenos, en condiciones que pueden simular las de campo.

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