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Influence of exogenously applied methyl jasmonate on soilborne pathogen, *Cylindrocarpon destructans in vitro*

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Abstract: Ginseng (*Panax ginseng*) is a traditional medicinal plant in China, and the dried root is highly valued for its medicinal properties and is widely used in Chinese traditional medicine. Cylindrocarpon root rot of ginseng caused by *Cylindrocarpon destructans* is the main disease, which is difficult to be eliminated from soil, leading to serious crop yield losses and limited the re-use of fields for successive ginseng crops. Methyl jasmonate (MeJA), which is one of the plant lipid derivatives, considered as a signaling substance during plant-microbe interactions. It is related to plant resistance to biotic and abiotic stress, can be involved in plant response to pathogens and other adversity stress and signal transmission, and can be used to induce plant disease resistance reaction. The objective of this study was to test the effects of MeJA on *C. destructans* and to find the possible relationships between MeJA and pathogenic factors of *C. destructans*. To assess the influence of MeJA on soil pathogens, the direct effects of artificially applied MeJA on *C. destructans* were evaluated, including the effects on colony growth, spore germination, biomass and phytopathogenic enzyme activities of this pathogen. The results showed that MeJA strongly inhibited its growth and spore germination, whereas it stimulated phytopathogenic enzyme activities. The colony diameter of *C. destructans* on PDA decreased from (8.23±0.15) cm (control) to (0.71±0.00) cm (800 μg · mL^{-1}). At the highest concentration (800 μg · mL^{-1}) of MeJA, the colony diameter of *C. destructans* had almost totally inhibited, but the diameter had no difference compared with the untreated control at lower concentrations (1-50 μg · mL^{-1} MeJA). The biomass reduced by 65.3%-100% and the percent of spore germination decreased by 100% at concentrations higher than 400 μg · mL^{-1}. Potent suppression of the growth of germ-tubes was observed at different concentrations, especially at (400-800) μg · mL^{-1}, where the growth of germ-tubes was inhibited completely. However, activities of phytopathogenic enzymes (pectinase, cellulase, amylase, and protease) were greatly stimulated by MeJA at higher concentrations (MeJA>200 μg · mL^{-1}), while the activity of protease was little changed. The activity of pectinase was high up to (0.61±0.05) U · mL^{-1} · min^{-1} at the concentration of 800 μg · mL^{-1}. The activity of cellulase was stimulated at high concentrations of MeJA in liquid culture, while it was suppressed at low concentrations (1-50 μg · mL^{-1}). The activity of cellulase was (0.31±0.02) μmol · min^{-1} at 800 μg · mL^{-1} of MeJA. Amylase activity substantial increased at concentrations of (200-800) μg · mL^{-1}, which was (0.45±0.02) μmol · min^{-1} at the concentration of 800 μg · mL^{-1}. It was concluded that MeJA greatly inhibited *C. destructans* growth and spore germination, but stimulated activities of hydrolytic enzymes of *C. destructans* at higher doses. There might be different mechanisms presented for the effects of MeJA on hyphal growth and

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virulence factors of *C. destructans*. The critical concentration of MeJA inhibitory effects on *C. destructans* was 200 μg · mL^{-1}. The research lays a foundation for the subsequent experiment using MeJA to induct disease resistance. This is of help to understand the mechanism of Cylindrocarpon root rot of ginseng and to control pathogen in practice.

Key words: methyl jasmonate, biomass, *Cylindrocarpon destructans*, pathogenic enzyme

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离体条件下外源茉莉酸甲酯对人参锈腐病菌的影响

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摘　要: 人参(*Panax ginseng*)是我国传统的名贵药材,由毁灭柱孢(*Cylindrocarpon destructans*)引起的人参锈腐病是严重影响人参产量和品质的重要根部病害之一,在人参生产中会造成严重的经济损失。茉莉酸甲酯(methyl jasmonate, MeJA)是一类新型的生长调节物质,既可以参与植物对病原菌及其他逆境胁迫做出的应答并进行信号传递,又可用来诱导植物的抗病反应。为了明确 MeJA 对人参锈腐病菌的影响并解析 MeJA 与病原菌致病因子之间的相互关系,该文研究了外源 MeJA 在不同浓度下对 *C. destructans* 的直接影响,包括对菌落生长、孢子萌发、菌丝生长量、病菌分泌水解酶的影响。结果表明:MeJA 能够强烈抑制病原菌的生长和孢子萌发,而对病原菌致病酶的活性则表现出促进作用;人参锈腐病菌在 PDA 平板上的菌落直径从(8.23±0.15) cm (对照) 减少到(0.71±0.00) cm (800 μg · mL^{-1} MeJA),在 MeJA 浓度达到最高时,菌落生长几乎完全被抑制;MeJA 的浓度大于 400 μg · mL^{-1}时,病原菌的生物量减少了 65.3%～100%,孢子萌发率和芽管长度减少了 100%;MeJA 在浓度大于 200 μg · mL^{-1}时,果胶酶、纤维素酶和淀粉酶活性升高而蛋白酶的活性却没有变化。综上表明,MeJA 对病原菌产生抑制作用的临界浓度为 200 μg · mL^{-1}。该研究结果为后续使用 MeJA 处理人参植株进行诱导抗病性的研究奠定了基础,同时也有助于进一步了解人参锈腐病的致病机理,并为病害防控提供了理论参考。

关键词: 茉莉酸甲酯, 生物量, 人参锈腐病菌, 致病酶

Ginseng (*Panax ginseng*) widely cultivated as a medicinal herb is an economically important cash crop in Northeast China (Wang, 2001). The dried root is highly valued for its medicinal properties and is widely used in Chinese traditional medicine (Rahman & Punja 2005a; Ali et al,2006). *Cylindrocarpon destructans* (teleomorph: *Nectria radicicola*), a pathogenic fungus responsible for Cylindrocarpon root rot of ginseng, is difficult to be eliminated from soil (Reeleder & Brammall, 1994; Punja, 1997). Cylindrocarpon root rot is one of the major threats to stable ginseng production (Reeleder & Brammall, 1994; Punja, 1997; Ahn & Lee, 2001; Rahman & Punja, 2005b; Kim et al, 2009), which can result in yield losses of up to 25%-30% (Seifert et al, 2003; Kernaghan et al, 2007). The pathogen *C. destructans* is the most important soil-borne pathogen that caused root rot of ginseng, limiting the re-use of fields for successive ginseng crops (Reeleder & Brammall, 1994; Reeleder et al, 2002).

Much attention has been paid to the effects of *C. destructans* on ginseng and other plant hosts, but much less to the effects of host plants on the pathogen in the plant-microbe interactions. In fact, pathogen invasion is closely related to host aspects. Jasmonate (JA) is widely distributed in the plant kingdom with multiple physiological functions during plant development, growth, and defense responses (Creelman & Mullet, 1997). Methyl jasmonate (MeJA), one of the major physiological active forms of jasmonates, is a vital cellular regulator that mediates diverse developmental processes in plants. It has been demonstrated to alter defense responses against biotic and abiotic stresses in various plant species (Penninckx et al, 1998). Large amounts of work has been done on the ability of MeJA to elicit plant defenses against necrotrophic fungi. Previous results show that MeJA can protect spruce seedlings a-

gainst the soil-borne pathogen *Pythium ultimum* (Kozlowski et al, 1999), and MeJA applied to potato leaves can induce systemic resistance against *Phytophthora infestans* (Cohen, 1993). Gaige (2010) suggested that MeJA and ethylene could induce partial resistance in *Medicago truncatula* against the charcoal rot pathogen *Macrophomina phaseolina*. The effects of MeJA on the control of Monosporascus root rot and vine decline of melon have also been studied (Aleandri et al, 2010). However, little research has been devoted to the direct effects of MeJA on specific pathogen itself e.g. on colony growth, spore germination, germ tube lengths, mycelial mass production, activities of pectinase, cellulase, amylase and protease of *in vitro* soil-borne pathogen, *C. destructans*.

The aim of this work is to assess the effects of MeJA on *Cylindrocarpon destructans* and to investigate *in vitro* whether there is a relationship between MeJA and the pathogenic factors of *C. destructans*.

1 Materials and Methods

1.1 Experimental materials

Cylindrocarpon destructans was isolated from infected ginseng roots from the major ginseng cultivation areas, by the laboratory of plant disease epidemiology, Shenyang Agricultural University, China. Colonies were cultured on potato dextrose agar (PDA) plates and grown at 20 ℃ in the dark in an incubator for 2 weeks (Rahman & Punja, 2005b, 2006). MeJA used in the experiment was obtained from the Sigma Co. (St. Louis, MO, USA).

1.2 Experimental methods

To determine the effects of MeJA on colony growth, MeJA was added to PDA to achieve the desired concentrations. Petri dishes containing PDA were inoculated with a 7 mm diameter mycelial plug from a 14-day-old culture of *C. destructans* and incubated at 20 ℃ for 2 weeks. Colony diameter was measured at 3-day intervals by taking two perpendicular measurements on each colony. Three replicate dishes of each treatment were carried out and the experiment conducted twice.

Percent germination and germ tube lengths were determined for spores of *C. destructans* in MeJA solution, following methods described by He & Wolyn (2005). Spores (1×10^6 spores · mL^{-1}) were harvested from the plates by rubbing the surface mycelium gently with a rubber swab and collecting the spores in distilled water. Spore suspension (4 mL) was diluted with 4 mL MeJA solution for each treatment and the resulting suspensions were incubated at 20 ℃ for 8 h. At least 100 spores per treatment replicate were measured microscopically for percent spore germination and germ tube length. The experiment was repeated twice with three replications and the data averaged.

The mycelial mass production was assessed by adapting the method of Rahman & Punja (2006) with minor modifications. Briefly, flasks containing 100 mL of potato dextrose broth were inoculated with a 7 mm diameter mycelial plug from a 14-day-old colony of *C. destructans* and incubated on a rotary shaker (130 r · min^{-1}) at 20 ℃ for 2 weeks. The mycelial mass (dry weight) from three replicate flasks was determined after filtration and drying at 80 ℃ for 12 h. The experiment was performed twice. Culture filtrate was centrifuged at 8 000 r · min^{-1} for 10 min at 4 ℃ and the supernatant was used for enzyme assays.

Pectinase activity (mainly polygalacturonase) was determined described by Silva et al(2005). One unit of enzyme activity was defined as the amount of β-galacturonic acid hydrolyzed from pectin per minute under the assay condition. Cellulase activity was assayed using the DNS (3,5-dinitrosalicylic acid) method (Berlin et al, 2005). One unit of cellulase activity was defined as the amount of enzyme that produced 1 μmol reduced sugar per minute. Amylase activity was determined by the procedure according to Murado et al(1997). One unit of amylase activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute. The gelatin assay of Tseng & Mount (1974) was used to quantify protease activity. One unit of protease activity was defined as the amount of enzyme causing an increase in absorbance of 0.01 in 1 min at 280 nm. The protein concentration in enzyme preparations was measured by the method of Lowry et al (1951) following precipitation with trichloroacetic acid.

1.3 Data analysis

Experiments were carried out using eight concentrations of MeJA: 0, 1, 10, 50, 100, 200, 400 and 800 μg · mL^{-1}. Data on the colony growth were analyzed by analysis of variance (ANOVA). Means of the treatments were compared by Duncan's multiple range tests at $P<0.05$. All statistical analyses were conducted with SPSS Base Version 11.5 statistical software (SPSS Inc. Chicago, IL).

2 Results and Analysis

2.1 Effects of MeJA on colony growth and mycelial mass production of *Cylindrocarpon destructans*

MeJA, a methyl ester of JA, plays an important role in the defense of plants against pathogens (Preston et al, 2001; Aleandri et al, 2010; Gaige et al, 2010). It can serve as a signal molecule bridging pathogen and plant host, particularly in the ginseng-*C. destructans* interactions. In the present study, the growth of *C. destructans* was strikingly suppressed by MeJA both in a potato dextrose liquid culture and on PDA plates. The dry weight of mycelia decreased from (74.00±9.54) mg (control) to 0 (800 μg · mL^{-1} MeJA) (Table 1).

A severe repression of colony growth on PDA was observed at a high concentration of MeJA, in which the colony diameter was found to be (5.54±0.23) cm at a concentration of 400 μg · mL^{-1} and (0.71±0.00) cm at a concentration of 800 μg · mL^{-1}, although the diameter had no difference compared with the untreated control [(8.23±0.15) cm] at lower concentrations (1−50 μg · mL^{-1} MeJA) (Table 1). This was in agreement with the report that MeJA inhibited mycelial growth of Phytophthora infestans *in vitro* (Cohen, 1993). MeJA was not significantly inhibitory to *C. destructans* at lower concentrations, but a potent suppression of colony growth was observed at high concentrations of MeJA (Table 1).

2.2 Effects of MeJA on spore germination and germ tube lengths

Dramatic inhibition of spore germination and germ tube growth by MeJA were obtained in a concentration-dependent manner. The percent of spore germination was strongly suppressed, with a reduction of 7.9% − 100.0% compared with the control (Table 2). Potent suppression of the growth of germ-tubes was observed at all concentrations (1−800 μg · mL^{-1}), especially at 400−800 μg · mL^{-1}, where the growth of germ-tubes was inhibited completely (Table 2).

Table 1　Effects of exogenous MeJA on mycelial growth and mycelial production of *Cylindrocarpon destructans* on potato dextrose agar (after 12 d)

MeJA concentration (μg · mL^{-1})	Colony diameter (cm)	Mycelial production (mg)
0	8.23±0.15a	74.00±9.54a
1	8.13±0.08ab	73.67±6.66a
10	8.09±0.1ab	66.67±7.37a
50	8.13±0.27ab	71.00±1.00a
100	8.08±0.14ab	70.00±13.89a
200	8.01±0.11ab	69.33±1.53a
400	5.54±0.23c	25.67±5.13b
800	0.01±0.00d	0.00±0.00c

Table 2　Effects of exogenous MeJA on spore germination and germ tube lengths of *Cylindrocarpon destructans* on potato dextrose agar (after 12 d)

MeJA concentration (μg · mL^{-1})	Percentage germination (%)	Germ tube length (μm)
0	76.33±11.55a	52.57±11.85a
1	70.33±10.98ab	32.35±3.80bc
10	68.44±6.64b	28.00±4.36cd
50	40.67±8.08c	33.18±3.97bc
100	43.00±9.77c	38.23±4.15b
200	21.22±4.99d	21.02±3.17d
400	0.00±0.00e	0.00±0.00e
800	0.00±0.00e	0.00±0.00e

2.3 Effects of MeJA on the activities of enzymes related to pathogenesis

Increase of the pectinase activity was observed with treatment by MeJA. The activity of pectinase was increased by MeJA depending on its concentration, with the maximum value [(0.61±0.05) U · mL^{-1} · min^{-1}] at the concentration of 800 μg · mL^{-1} (Table 3). The ac-

tivity of cellulase was stimulated at high concentrations of MeJA (200–800 μg · mL^{-1}) in liquid culture, while it was suppressed at low concentrations (1–50 μg · mL^{-1}). The activity of cellulase was (0.31 ± 0.02) μmol^{-1} · min^{-1} at the highest concentration (800 μg · mL^{-1}) of MeJA (Table 3). At lower concentrations of MeJA (1 – 100 μg · mL^{-1}), amylase activity little changed, but substantial increase of the activity was found at high concentrations of 200 – 800 μg · mL^{-1}, which was (0.45±0.02) μmol^{-1} · min^{-1} at the concentration of 800 μg · mL^{-1} (Table 3). Protease activity by *C. destructans* had scarcely influenced by MeJA in liquid culture, although small amounts of fall tendency was observed, which the activity was almost no difference compared to control (Table 3).

Table 3 Effects of MeJA at different concentrations on hydrolytic enzymes related to pathogenesis of *Cylindrocarpon destructans* in a liquid culture (after 12 d)

MeJA Concentration (μg · mL^{-1})	Pectinase activity (U · mL^{-1} · min^{-1})	Cellulase activity (μmol · min^{-1})	Amylase activity (μmol · min^{-1})	Protease activity (U · mL^{-1} · min^{-1})
0	0.41± 0.01a	0.13± 0.03b	0.28± 0.01a	3.35± 0.10a
1	0.48± 0.05a	0.10± 0.03b	0.24± 0.01a	3.35± 0.16a
10	0.43± 0.01a	0.08± 0.00ab	0.25± 0.01a	3.32± 0.05a
50	0.42± 0.03a	0.07± 0.00a	0.24± 0.01a	3.24± 0.12a
100	0.51± 0.08b	0.07± 0.00a	0.26± 0.03a	3.12± 0.03a
200	0.50± 0.04b	0.21± 0.01c	0.43± 0.04b	3.28± 0.52a
400	0.55± 0.03bc	0.25± 0.03cd	0.42± 0.02b	3.46± 0.15a
800	0.61± 0.05c	0.31± 0.02d	0.45± 0.02b	3.31± 0.24a

3 Discussion

It is well known that spore germination and mycelial growth of *C. destructans* play an important part in the infection process in plant diseases. We believed that decreased germination and mycelial growth of *C. destructans* by MeJA would be one of the mechanisms on plant resistance to pathogens. From the present study, MeJA not only enhances the plant resistance to pathogens but also directly inhibits the growth of the pathogens.

Enzymes related to pathogenesis secreted by *C. destructans*, such as pectinase, cellulase, amylase and protease, were important pathogenic factors in the progression of the infection. Pectinases and cellulases facilitate the penetration of the fungus into the plant by the hydrolytic cleavage of polymers (pectic substances, cellulose) which constitute the plant cell walls (Fuchs et al, 1965). It has been proposed that proteases may be required for nutritional purposes or to degrade protein in the plant cell wall to allow spread of the pathogens or overcome host defenses (Dow et al, 1990). Increase of amylase activity from the fungi contributes to the deposition and utilization of host carbon source. In the current study, pectinase, cellulase and amylase activity of *C. destructans* was stimulated by MeJA. Pectinase activity at the highest concentration of MeJA increased by 47.7%. Cellulase activity was repressed by MeJA at concentrations lower than 100 μg · mL^{-1}, while was stimulated at high concentrations (200–800 μg · mL^{-1}). A great increase of amylase activity was obtained treated with MeJA at concentrations higher than 200 μg · mL^{-1}, which was increased by 63% at the concentration of 800 μg · mL^{-1}. Little effect of MeJA on *C. destructans* protease activity was found (Table 3). The findings meant that excessive MeJA artificially added in practice would have adverse effect on the plant, which needs to be further studied in the future.

In conclusion, MeJA inhibited the colony growth and spore germination of *Cylindrocarpon destructans*, while at the same time stimulated the production of phytopathogenic enzymes. The critical concentration of MeJA inhibitory effects on *C. destructans* was 200 μg · mL^{-1}. The results lays a foundation for the subsequent experiment using MeJA to induct disease resistance.

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