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Note

Production of Salicylic Acid in Tobacco and Cowpea Plants by a Systemic Fungicide Ferimzone and Induction of Resistance to Virus Infection

Masaharu NAKAYAMA, Kazuho MATSUURA and Tetsuro OKUNO*

Agricultural Research Laboratories, Agro Division, Takeda Chemical Industries, Ltd., Wadai, Tsukuba 300-42, Japan
**Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*

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INTRODUCTION

Plants have a number of defense mechanisms against pathogen attacks. In addition to a cultivar-race specific resistance, plants can acquire local and systemic resistance following infection with various pathogens which induce necrotic lesions.[1,2] Systemic acquired resistance (SAR) is effective to a wide range of plant pathogens including fungi, bacteria and viruses[3,4] and it also can be induced by chemicals such as salicylic acid (SA) and its derivatives,[5-7] 2,6-dichloroisonicotinic acid (INA)[8] and 7-methoxycarbonyl benzo-1,2,3-thiadiazol[9] that have no antimicrobial activity *in vitro*. Although it remains to be investigated how these chemicals are involved in SAR, infection with necrotizing pathogens increase endogenous SA levels in plants. Recent reports suggest that SA serves as an endogenous signal molecule required for the induction of SAR.[10-12]

Ferimzone, (Z)-2′-methylacetophenone 4,6-dimethylpyrimidin-2-ylhydrazone (Fig. 1) is a novel systemic fungicide developed for the control of rice diseases and exhibits strong antifungal activity against many plant-pathogenic fungi including *Pyricularia oryzae* both *in vitro* and *in vivo*.[13-15] Since ferimzone was found to be effective for the control of some bacterial diseases in a field,[16] it has been tempting to test whether this chemical has SAR inducing activity or not. We report here that spraying tobacco and cowpea plants with ferimzone induced acquired resistance to infection with tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), respectively, and that the treatment with ferimzone significantly increased the amount of endogenous SA in both tobacco and cowpea plants.

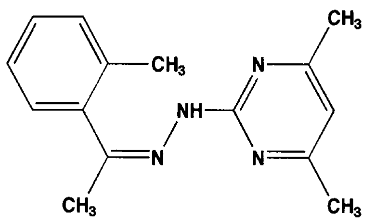

Fig. 1 Chemical structure of ferimzone.

MATERIALS AND METHODS

1. Plants

Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) and cowpea (*Vigna unguiculata* L. cv. Sanjakusasage) were grown in a plastic pot containing soil in a greenhouse at 25°C under natural day light.

2. Chemicals

Ferimzone (technical grade containing Z-isomer of more than 97.0%) was used throughout all the experiments. The compound was dissolved in ethanol and diluted with deionized water to make ethanol concentration less than 1% in a final solution. SA was dissolved in deionized water by adjusting pH to 7 with NaOH.

3. Viruses

TMV (OM strain) and CMV (Y strain) were propagated in *N. tabacum* L. cv. Bright Yellow and cv. Samsun NN, respectively and purified using the method described by Takanami & Tomaru.[17]

4. Extraction and Analysis of Salicylic Acid

Endogenous SA was extracted from tobacco and cowpea leaves sprayed with ferimzone. Leaves (1 g) were sliced, rolled into the bottom of test tubes and soaked with 10 ml of 2% acetic acid. The solution was boiled for 10 min and centrifuged for 10 min at $1000 \times g$, and the supernatants were used to assess the amount of SA in leaves. The SA extract was injected onto a C-18 column (II 5C18-HG, Wako Pure Chemical Industries, Ltd.) equilibrated with 50% aqueous methanol (v/v) containing 0.05% phosphoric acid and eluted at 1 ml/min. The peak of free SA was detected with a fluorescent detector (excitation 290 nm, emission 402 nm).

5. Virus Infection Assay

Two month-old tobacco plants at 6 to 8 leaf stage and 7 day-old cowpea plants were sprayed with ferimzone at the concentrations of 50 and 500 μM, with SA at the concentrations of 5 and 10 mM, or with water. All plant leaves were washed with tap water at the following day. Leaves of the plants were inoculated with TMV at the concentration of 0.2 μg/ml or CMV at the concentration of 3 μg/ml, 1, 4 and 7 days after chemical treatment. The number of local lesions was counted 3 days after inoculation.

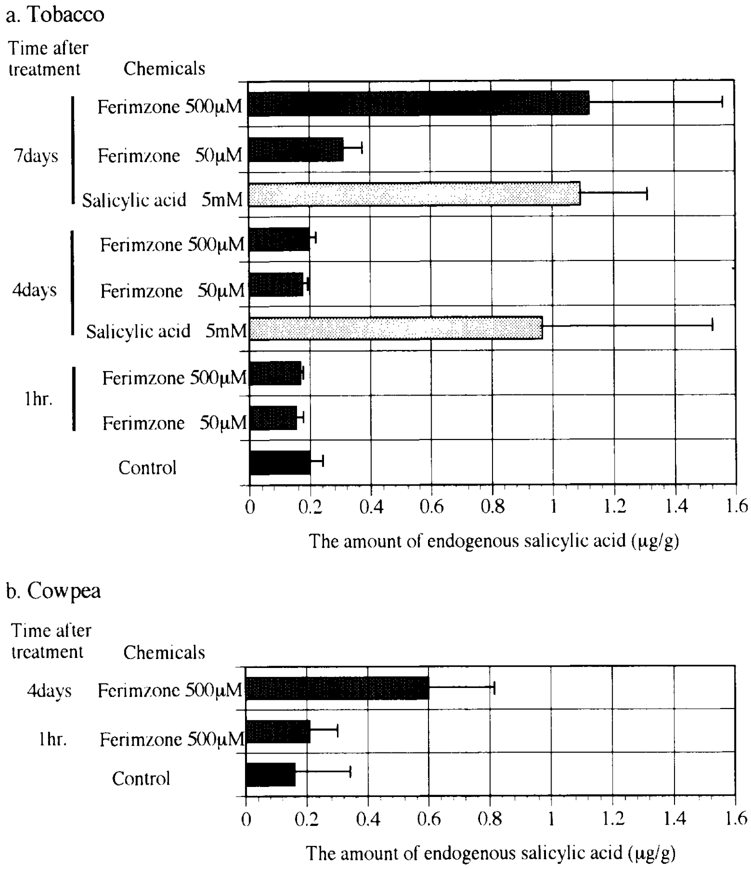

Fig. 2 The quantitative analysis of endogeneous salicylic acid extracted from leaves treated with ferimzone or salicylic acid. The means of amounts of endogeneous salicylic acid in 9 leaves from tobacco plants and 6 to 8 leaves from cowpea plants were calculated, respectively. Error bars show significative limits ($p=5\%$) with the Tukey compromise test.

RESULTS AND DISCUSSION

In tobacco leaves at 7 days and in cowpea leaves at 4 days after ferimzone treatment at the concentration of 500 μM, the amount of endogenous SA increased by 3 to 4 folds compared to water treated controls and it was comparable to that in leaves sprayed with SA at the concentration of 5 mM (Fig. 2). No significant differences in the amount of endogenous SA were observed between ferimzone-treated and water-treated leaves in both tobacco sampled 4 days and cowpea sampled 1 hr after the treatment. Ferimzone at the concentration of 50 μM did not significantly influence the amount of endogenous SA in tobacco plants.

Ferimzone was tested for the ability to influence local lesion formation by TMV on tobacco leaves by spraying at the concentration of 500 μM. The number of local lesions on either sprayed or unsprayed upper leaves significantly decreased compared to the water-treated controls when the leaves were inoculated with TMV 7 days after the treatment (Table 1, Expts. 1 and 2). However, no significant differences in the number of local lesions were observed between ferimzone-treated and water-treated leaves inoculated 4 hr and 1 day after the treatment (Table 1, Expts. 1 and 2). Similar results were obtained by spraying SA at the concentration of 5 mM (Table 1, Expt. 1). Significant reduction in the number of local lesion caused by CMV infection was also observed in cowpea leaves pretreated with ferimzone and SA (Table 1, Expts. 3 and 4). Ferimzone and SA at the concentrations up to 500μM and 5 mM did not cause any visible damage on leaves of tobacco or cowpea plants. However, when ferimzone and SA were applied at higher concentrations of 1 and 10 mM, respectively, they sometimes incited small necrosis spots on tobacco and cowpea leaves. Ferimzone caused severe necrosis and deterioration of leaves in tobacco at the concentrations higher than 5 mM.

The results presented in this report showed the ability of ferimzone to induce resistance against virus infection in both tobacco and cowpea plants several days after spray treatment. Since metabolic products of ferimzone in higher plants do not contain any signal molecules reported so far in reference to the activation of plant defense responses, we consider that ferimzone in itself has the ability to induce virus resistance in plants.[18] Much evidence has indicated that endogenous SA is involved in the activation of plant defense responses including virus resistance.[10-12] SA whose amount is increased in ferimzone-treated plants is likely responsible for the induction of virus resistance by ferimzone. Involvement of the metabolic products of ferimzone could not be excluded for the induction of endogenous SA in plants.

Ferimzone was originally developed for the control of

Table 1 The effect of ferimzone on local lesion formation by TMV and CMV in tobacco and cowpea leaves.[a)]

Expt.	Plant/Virus	Leaf position	Time between treatment and inoculation	Chemical concentration		Average number of lesions[b)]		% protection
1	Tobacco /TMV	Sprayed	4 hr	500 μM	Ferimzone	117±16		
				5 mM	Salicylic acid	91±11		
					Control	128±20	NS	
		Sprayed	7 days	500 μM	Ferimzone	34±7	a	58
				5 mM	Salicylic acid	34±3	a	58
					Control	80±10	b	
2	Tobacco /TMV	Sprayed	1 day	500 μM	Ferimzone	43±4		
					Control	71±16	NS	
		Unsprayed upper	1 day	500 μM	Ferimzone	64±19		
					Control	61±5	NS	
		Sprayed	7 days	500 μM	Ferimzone	13±3	a	65
					Control	37±4	b	
		Unsprayed upper	7 days	500 μM	Ferimzone	20±1	a	66
					Control	59±12	b	
3	Cowpea /CMV	Sprayed	4 days	500 μM	Ferimzone	74±21	a	88
				10 mM	Salicylic acid	135±46	ab	79
					Control	634±122	c	
		Sprayed	7 days	500 μM	Ferimzone	13±5	a	82
				10 mM	Salicylic acid	27±5	ab	62
					Control	71±11	c	
4	Cowpea /CMV	Sprayed	4 days	50 μM	Ferimzone	99±12	a	65
				5 mM	Salicylic acid	213±34	ab	24
					Control	279±76	b	

a) Leaves were inoculated with TMV (0.2 μg/ml) or CMV (3 μg/ml) at indicated time after chemical treatment.
b) Mean values per leaf from 8 to 20 tobacco leaves are presented in the experiments 1 and 2. Mean values per 2 primary leaves from 8 cowpea plants are presented in the experiments 3 and 4.
Figures with the same letter are not significantly different ($p=5\%$) according to Tukey compromise test. NS: not significant.

fungal diseases and has wide antifungal spectrum *in vitro*,[14,15,18] but has little antibacterial activity *in vitro*. The observed control activity of ferimzone in some bacterial diseases in field rice plants[16] might be explained by the ability of ferimzone to induce resistance response in plants reported here although there have been no report for the involvement of SA in defense response in rice plants. Ferimzone in itself or unknown substances including metabolites of ferimzone may function as a signal molecule for the induction of bacterial resistance in rice plants. We cannot exclude the possibility of the involvement of SA as a signal molecule in the induction of resistance in rice plants. The present results indicate that ferimzone has a property of resistance inducing chemical like INA in addition to the original fungicide activity.[8] Consequently, ferimzone may be used as a resistance inducing chemical to protect crops against fungal, bacterial and viral diseases.

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要 約

浸透性殺菌剤フェリムゾンによるサリチル酸はウイルス感染に対する抵抗性誘導能をもつ

中山政治，松浦一穂，奥野哲郎

フェリムゾン，(*Z*)-2′-methylacetophenone 4, 6-dimethylpyrimidin-2-ylhydrazone をタバコおよびササゲに処理した際には，内在性サリチル酸量の増加が認められた．そこで，フェリムゾン処理を行なった植物に，タバコモザイクウイルス(TMV)，あるいは，キュウリモザイクウイルス (CMV) の接種を行なったところ，サリチル酸処理の場合と同様な局部獲得抵抗性を示した．さらに，タバコの場合は TMV 接種に対して全身獲得抵抗性が確認された．これらの抵抗性は，それぞれの植物に対する TMV，あるいは，CMV 接種による局部病斑の 50% 以上の減少として観察された．フェリムゾンは植物体に内在性サリチル酸を誘導する作用性があり，フェリムゾン処理された植物体はこの内在性サリチル酸量の増加によって抵抗性を獲得することが示唆された．