シャリンバイの新規ファイトアレキシン・4'-methoxyaucuparinの生成と抗菌性

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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巻/号	57巻2号
掲載ページ	p. 232-238
発行年月	1991年4月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

日植病報 57: 232–238 (1991)
Ann. Phytopath. Soc. Japan 57: 232–238 (1991)

Accumulation and Antifungal Spectrum of 4′-Methoxyaucuparin as a New Phytoalexin in *Rhaphiolepis umbellata* Makino*

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Abstract

Accumulation and antifungal activity of a newly isolated phytoalexin, 4′-methoxyaucuparin, were described. The phytoalexin was accumulated in the leaves of *Rhaphiolepis umbellata* Makino inoculated with *Pestalotiopsis* sp. or *Entomosporium mespili*. Accumulation of 4′-methoxyaucuparin also occurred with treatment of 10^{-3} M NaN_3 or $HgCl_2$ as abiotic elicitors. Of the elicitors tested, $HgCl_2$ was found to be the most effective, being able to induce 4′-methoxyaucuparin at the concentration of 4.385 $\mu g\ g^{-1}$ fresh weight. The inhibitory action of 4′-methoxyaucuparin against *Pestalotiopsis* sp., pathogenic fungus of *R. umbellata*, was weaker than that against non pathogenic fungi.

(Received August 6, 1990)

Key words: Rosaceae, *Rhaphiolepis umbellata*, phytolexin, 4′-methoxyaucuparin.

INTRODUCTION

In 1980 Morita *et al.*[13] reported on the production of a new phtyoalexin in the cortical layer of loquat shoot (*Eriobotrya japonica* L., Rosaceae) when it was inoculated with *Colletotrichum lindemuthianum*. On our cooperative study with Watanabe[17] the phytoalexin was identified as aucuparin which was previously known as a normal constituent of healthy *Sorbus aucuparia* L. (Japanese name 'nanakamado'), Rosaceae[6]. In addition, eriobofuran as another new phytoalexin was also found in loquat inoculated with *Entomosporium mespili*[12]. At least, on the other hand, α,β,γ-pyrufuran from perry pear trees (*Pyrus communis* L.)[7,8], cotonefuran from *Cotoneaster lactea* W.W. Sm[3] and pentacyclic triterpene from *Malus pumila* Mill.[9] were reported as phytoalexins from family of Rosaceae other than loquat.

In our current study on Rosaceae phytoalexins, we succeeded in isolating and characterizing of a new biphenyl phytoalexin, 3,5,4′-trimethoxy-4-hydroxybiphenyl (4′-methoxyaucuparin), from the leaves of *Rhaphiolepis umbellata* Makino (Japanese name 'sharinbai') stressed by the treatment with abiotic elicitors[18]. The present paper describes the results of experiments on the accumulation of 4′-methoxyaucuparin in the leaves of *R. umbellata* and on further antifungal activities of this phytoalexin.

MATERIALS AND METHODS

Plant material. Seedlings of *Rhaphiolepis umbellata* were grown in a glass house

* Part of this work was reported at the Meeting of Phytopathological Society of Japan, Matsuyama, April, 1990.

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at about 25°C for six months before treatment with several elicitors.

Fungi. Two pathogenic fungi, *E. mespili* and *Pestalotiopsis* sp., were isolated from *R. umbellata*. Four non-pathogenic fungi, *Alternaria kikuchiana*, *Ascochyta pisi*, *Botrytis cinerea* and *Monilinia fructicola* were isolated from *Pyrus serotina* Rehd., *Pisum sativum* L., *Rosa multiflora* Thunb. and *Prunus avium* L., respectively. These isolates were maintained on potato sucrose agar (PSA) at 25°C in the dark and transferred periodically to fresh medium in the laboratory.

Phytoalexin accumulation. To elicit 4′-methoxyaucuparin by fungal infection, leaves of *R. umbellata* were inoculated with spore suspensions of *Pestalotiopsis* sp. and *E. mespili*. The spores of *Pestalotiopsis* sp. were obtained by flooding the surface of sporulating cultures with sterilized water and rubbing gently with a glass rod. Spore suspensions were filtered through cheese-cloth under sterile conditions. The spores of *E. mespili* were prepared from diseased leaves of *R. umbellata* with sporulating lesions. The spore suspensions of the two fungi were adjusted to 10^5 spores per ml. Aqueous solution of 10^{-3} M NaN_3 or $HgCl_2$ was used as abiotic elicitors.

For elicitation of 4′-methoxyaucuparin in the attached leaves, whole plants were sprayed with the spore suspensions or abiotic elicitors and were kept under humid condition in the dark at 25°C for 72 hr before transfer to the glass house. The accumulation of the phytoalexin in the leaves was checked periodically.

The accumulation of 4′-methoxyaucuparin in detached leaves was also examined. The solution of abiotic elicitors or spore suspension of *Pestalotiopsis* sp. were dropped on the upper (adaxial) leaf surfaces, respectively, and the leaves were incubated in the dark at 25°C under humid condition. After 24 hr the phytoalexin production was estimated.

Extraction and purification of the phytoalexin. Ten g fresh weight of plant leaf samples were extracted with 80% aqueous ethanol (100 ml) for 24 hr at 25°C. The ethanolic extract was filtered through two layers of cheese-cloth and then evaporated to 10 ml at 35°C under reduced pressure. The condensed extract was added with 90 ml of water and centrifuged ($9{,}000 \times g$; 30 min). The supernatant was adjusted to pH 3.0 with HCl and extracted with an equal amount of petroleum ether (3 times). The combined petroleum ether phases were evaporated to dryness at 35°C. The residue was dissolved in 1 ml ethanol, and the sample was applied to a silica gel TLC plate (Merck GF_{254}, 0.25 mm) and eluted with the solvent consisted of hexane : ethyl acetate : methanol (60:40:1, v/v). Detection of the phytoalexin on the plate was done by using bioassay method as described by Miyakado *et al.*[12] using *Cochliobolus miyabeanus* as test organism. The fungitoxic fractions were then collected, combined, dried at 35°C under reduced pressure, and dissolved in a small volume of ethanol.

For further purification, a Waters 6,000 A pump system equipped with a Waters U6K universal injector was used for analytical or preparative high performance liquid chromatography (HPLC). Chromatographic and spectral data from the eluate were acquired with a Jasco UV detector. Samples in ethanol were filtered through a filter of pore size 0.45 μm (chromatodisc 4A filters) and then the aliquot samples (20 μl) were injected on a LiChoCART column (125 × 4 mm, 5 μm particle size) and eluted with methanol : water (65:35 v/v; 0.5 ml/min). Under these conditions the active compound had a retention time of 7.8 ± 0.2 min.

Quantitative determination of the phytoalexin. Plant samples (1 g of fresh weight) were extracted with 10 ml of ethanol as described above. The dried residue obtained after evaporation was partitioned with distilled water. The supernatant was adjusted to pH 3.0 with HCl and extracted with an equal amount of petroleum ether (3 times). Petroleum ether fractions were combined and evaporated to dryness. The residue was dissolved in 1 ml ethanol.

Samples in ethanol were filtered through a filter of pore size 0.45 μm. A solution of 10 μl of the samples was chromatographed through a LiChoCART column and elution mentioned above. Ten μl of pure phytoalexin (20 μg/ml) was injected under the same conditions as a

standard.

Bioassay of antifungal activity. The purified phytoalexin was assayed for toxicity by measuring its effect on the spore germination and hyphal elongation of *Pestalotiopsis* sp., pathogenic fungi, and four non-pathogenic fungi with a method as described by Király *et al.*[10] An ethanolic solution of 4′-methoxyaucuparin was added to water so that the final volume of ethanol was 2%. A drop of the solution was added to water agar block containing spore suspensions (10^5 spores/ml) and then incubated at 25°C. The germination rate and germ tube length were measured by using a light microscope.

RESULTS

Characteristics of phytoalexin

The characteristics of the phytoalexin were the same as those described in the previous report[18]. The λ_{max}^{EtOH} nm of the phytoalexin was 268 nm (Fig. 1). The high-resolution MS of the compound gave a molecular ion at *m/e* 246 and the molecular formula was $C_{15}H_{16}O_4$ (Fig. 2)[18].

Using hexane : ethyl acetate : methanol (60:40:1, v/v) as the developing solvent for TLC, a highly absorbing spot under UV light (365 nm) was observed at *Rf* 0.60. This spot coincided with a strong inhibition area in the plate culture test.

Phytoalexin accumulation in attached leaves

Usually no fungitoxic compound was detected in healthy leaves of *R. umbellata* (Fig. 3).

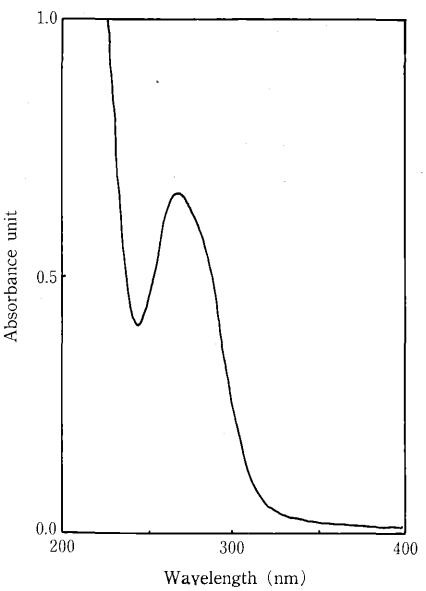

Fig. 1. UV spectrum of 4′-methoxyaucuparin in ethanol.

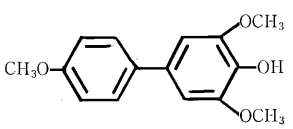

Fig. 2. The structure of 4′-methoxyaucuparin (Watanabe *et al.*, 1990).

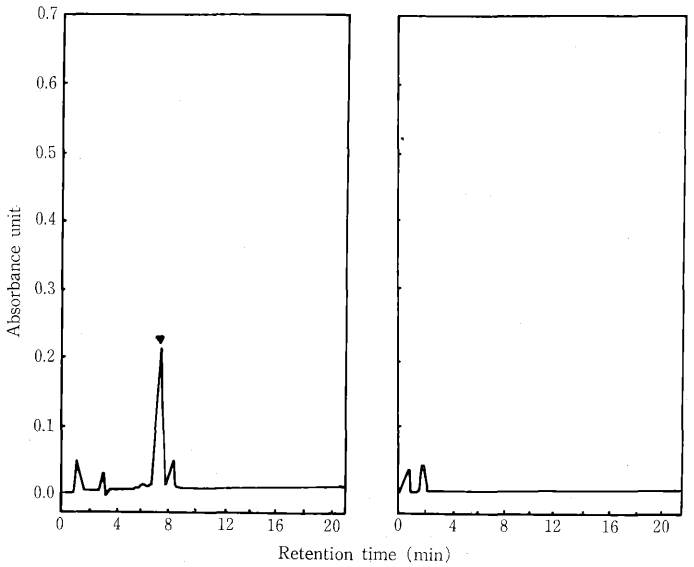

Fig. 3. Chromatoscans at 266 nm of 4′-methoxyaucuparin from attached leaves of *Rhaphiolepis umbellata*. Treated by NaN_3 for 2 days: left chromatoscans. Non treated control: right chromatoscans. Elution gradient (water/methanol) is described in text. The arrow indicates 4′-methoxyaucuparin. The peak was not detected in the non-treated.

Table 1. Accumulation of 4′-methoxyaucuparin on detached and attached *Rhaphiolepis umbellata* leaves treated with various elicitors

Elicitor	Attached (A) or detached (D) leaf	4′-methoxyaucuparin (μg/g fresh weight) [b]	
		Non-treatment	Treatment
Entomosporium mespili	A	0.021	3.072
Pestalotiopsis sp.	A	ND [a]	2.614
Pestalotiopsis sp.	D	0.015	0.303
10^{-3} M $HgCl_2$	A	0.011	4.385
10^{-3} M $HgCl_2$	D	0.025	1.860
10^{-3} M NaN_3	A	ND	1.825
10^{-3} M NaN_3	D	0.013	0.906

a) ND: not detected.
b) Values are expressed as μg/g 4′-methoxyaucuparin 6 days after treatment. Experiments were repeated three times with triplicated samples per each experiment.

In some cases, however, minute amounts of the compound could be detected (Table 1). Inoculation with *Pestalotiopsis* sp. on attached leaves induced 4′-methoxyaucuparin accumulation of 2.614 μg/g fresh weight under optimum condition. The most active elicitor for 4′-methoxyaucuparin in attached leaves was 10^{-3} M $HgCl_2$ which led to phytoalexin accumulation of 4.385 μg/g fresh weight whereas NaN_3 induced that of 1.825 μg/g (Table 1).

Phytoalexin accumulation in detached leaves

4′-Methoxyaucuparin was accumulated in detached *R. umbellata* leaves by the inoculation with *Pestalotiopsis* sp. as well as by the treatment with $HgCl_2$ or NaN_3 (Table 1). The accumulation was clearly found 2 days after treatment and it remained stably for about 6 days and then declined during the next 6 days (Fig. 4). Further result showed that the accumulation in detached leaves was lower than that in attached leaves.

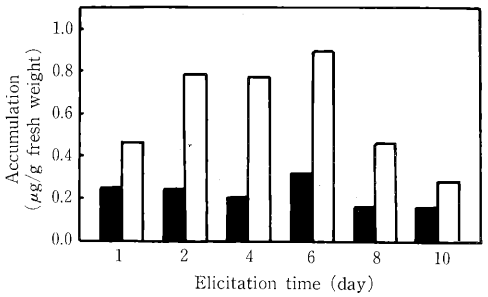

Fig. 4. Accumulation of 4′-methoxyaucuparin from detached leaves of *Rhaphiolepis umbellata* treated with spores of *Pestalotiopsis* sp. (■) and NaN_3 (□) at different elicitation times.

Table 2. Effect of 4′-methoxyaucuparin on the germination and hyphal growth of five plant pathogenic fungi

Fungus (host plant)	Germination rate (%)[a]			Germ tube length (μm)[b]		
	Concentration of 4′-methoxy-aucuparin (μg/ml)			Concentration of 4′-methoxy-aucuparin (μg/ml)		
	0[c]	10	40	0	10	40
Pestalotiopsis sp. (*Rhaphiolepis umbellata*)	93 (100)[d]	98 (105)	60 (64)	37 (100)	34 (91)	22 (60)
Alternaria kikuchiana (pear)	83 (100)	47 (57)	33 (40)	16 (100)	9 (56)	5 (31)
Ascochyta pisi (pea)	89 (100)	48 (54)	12 (13)	13 (100)	7 (57)	4 (30)
Botrytis cinerea (rose)	92 (100)	25 (27)	6 (7)	15 (100)	3 (20)	2 (13)
Monilinia fructicola (cherry)	88 (100)	55 (63)	23 (26)	35 (100)	18 (51)	6 (17)

a) Three hundred spores were counted.
b) Mean length of germ tubes was calculated on 150 spores, respectively.
c) Control (treated with 2% ethanol only).
d) Number in parentheses represents an exponent against the number of the control (100).

In vitro antifungal activity of the phytoalexin

4′-Methoxyaucuparin prevented both spore germination and hyphal growth of fungi tested at the concentration of more than 40 μg/ml. The solvent (ethanol 2%, v/v) had no significant effect. 4′-Methoxyaucuparin at the concentration of 10 μg/ml inhibited spore germination and hyphal growth of non-host pathogenic fungi while at the same concentration it had no effect on the spore germination and hyphal growth of *Pestalotiopsis* sp., pathogenic to *R. umbellata* (Table 2).

DISCUSSION

Extensive studies of phytoalexins in several plant families have showed that their phytoalexins are chemically heterogeneous group of compounds but certain chemical types tend to be associated with particular plant families[5,11]. 4′-Methoxyaucuparin, the phytoalexin isolated from *R. umbellata*, was found to be related to aucuparin which was isolated from other member of Rosaceae, namely *E. japonica*. However, 4′-methoxyaucuparin differed in molecular formulae, UV λ_{max} in EtOH and molecular weight from aucuparin. In general from their chemical structures, it is suggested that phytoalexins of Rosaceae plants such as loquat, perry pear trees, *C. lactea*, apple and *R. umbellata* usually are diphenyl and dibenzofuran compounds.

When challenged with biotic or abiotic elicitors, *R. umbellata* leaves accumulated a fungi-

toxic compound which was consistent with Paxton's definition of phytoalexins[15]. This compound was not usually present in healthy tissues. The present data are in agree with those of previous works which showed that some times in healthy or untreated plant a minute amount of phytoalexin could be detected even when microbial contamination was prevented[1,16]. In this experiment abiotic and biotic elicitors differed in their capacity to accumulate 4′-methoxyaucuparin in *R. umbellata*. In detached leaves, abiotic elicitor could induce 4′-methoxyaucuparin higher than biotic one. However in attached leaves NaN_3 induced 4′-methoxyaucuparin lower than biotic elicitor. Role of elicitors to induce the accumulation of 4′-methoxyaucuparin is yet to be studied.

Most phytoalexins have been successfully elicited with high yields in the active tissues (leaves, pods) incubated in good aeration at between 15–30°C[2]. Our results using *Pestalotiopsis* sp. also showed that accumulation of 4′-methoxyaucuparin in the detached leaves was about ten times lower than that in attached leaves (Table 1). The low level of phytoalexin accumulation in the detached leaves was probably due to the rapid decreasing in the physiological activity of the detached leaves in the laboratory condition, because the maximum accumulation of the phytoalexin occurred 6 days after treatment and then descreased rapidly thereafter.

We found that 4′-methoxyaucuparin inhibited either pathogenic or non-pathogenic fungi tested, but the inhibition activity of the compound to pathogenic fungi was lower than that to non-pathogenic fungi. These results were capable of the conception of previous workers who postulated that pathogenic fungi were relatively insensitive to their host's phytoalexins, whereas non-pathogens were sensitive[4,14].

Thanks are due to Mr. M. Mitsutomi of the Laboratory of Food Chemistry, Faculty of Agriculture, Saga University for his technical assistance and to Asahi Glass Foundation for the scholarship given to the first author, respectively.

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和 文 摘 要

Siti Muslimah WIDYASTUTI・野中福次・渡辺敬介・丸山英子・佐古宣道：シャリンバイの新規ファイトアレキシン・4′-methoxyaucuparin の生成と抗菌性

シャリンバイの葉にシャリンバイ白斑病菌およびビワごま色斑点病菌の胞子を接種すると，ファイトアレキシンの1種が生成され，これは新規の 4′-methoxyaucuparin であることが確認された。この 4′-methoxyaucuparin はまた，10^{-3} M NaN_3 および $HgCl_2$ をエリシターとして用いても生成されたが，10^{-3} M $HgCl_2$ による生成量が最も多く，生重 1 g 当り 4.385 μg であった。4′-methoxyaucuparin の抗菌性について検討した結果，シャリンバイの病原菌である白斑病菌よりも非病原菌を強く阻害した。