Aconitic Acid Content of Some Cereals and Its Effect on Aphids

Maqsood A. RUSTAMANI, Katsuo KANEHISA¹
and Hisaaki TSUMUKI

Research Institute for Bioresources, Okayama University, Kurashiki 710, Japan

(Received April 6, 1991; Accepted July 29, 1991)

Aconitic acid was observed to be a prevailing substance in acid-soluble components of wheat (8 lines), corn (10 lines), sorghum (2 lines) barnyard grass (3 subspecies) and rice (1 line). In barley it was present in rather small and inconsistent amounts (8 lines). We studied possible relationships between aconitic acid and degree of resistance or susceptibility to aphids. Corn, a line of sorghum and a mature stage of a subspecies of barnyard grass contained between 200 and 600 $\mu\text{g/g}$ of aconitic acid/g, and seemed to be highly resistant. Between 100 to 200 $\mu\text{g/g}$ were found in other subspecies of barnyard grass, a rice line and a susceptible line of sorghum. Ten to 25 $\mu\text{g/g}$ were found in wheat. Some lines of wheat showed weak resistance while others were susceptible. Aconitic acid may play a role in the resistance of some cereals to aphids.

Key words: Aconitic acid, cereal plants, resistance to aphids

INTRODUCTION

Resistance of cereal plants to insects is related to the presence of secondary metabolites that may be toxic or deter feeding, as has been reported for gramine alkaloid in barley against aphids (CORCUERA, 1984; KANEHISA et al., 1990); DIMBOA in corn against the European corn borer (KLUN et al., 1967); hydroxamic acid including DIMBOA in wheat against aphids (ARGANDONA et al., 1980; LESZCZYNSKI and DIXON, 1990); Duhrrin alkaloid, *p*-hydroxybenzaldehyde and procyanidin in sorghum against aphids (DRYER et al., 1981); and (*E*)-aconitic acid in barnyard grass to the brown plant-hopper (KIM et al., 1976).

We separated secondary metabolites in several cereals by liquid chromatography and tried to correlate these data to those of an aphid resistance survey. We observed that the relatively large amounts of aconitic acid present in most of the cereals tested possessed resistant activity. This report quantitatively describes the aconitic acid contents of cereals and their effect on aphids.

MATERIALS AND METHODS

Cereal plants. Eight lines of wheat, *Triticum aestivum* L. (Table 1) and 10 lines (Table 2) of barley, *Hordeum vulgare* L. (including resistant and susceptible lines) were

¹ To whom all correspondence should be addressed.

obtained from the plant genetics laboratory at our Institute and sown in a corner of a 2 ha field in mid-November. About 200 tillers per line were cultured. Ten lines (Table 3) of corn, *Zea mays* L., were obtained from the Hokkaido Agricultural Experimental Station and sown in greenhouse pots at the end of April. Seedlings about 15 cm high were transplanted to the field in the middle of May (usually 5 tillers per line), and harvested in August. Resistant (PE-954177) and susceptible (Redlan-B) lines of sorghum, *Sorghum bicolor* MOENCH, were obtained from the Chugoku Agricultural Experimental Station and sown in the field in early May (20 tillers per line). Three barnyard grasses, *Echinochloa oryzicola* VASING, *E. crus-galli* BEAUV. and *E. crus-galli* var. *formosensis* OHWI, and a rice line, Akebono, from our Institute were cultured in a rice field using normal cultivation procedure.

Extraction. Five or 10 g of well-developed leaves (usually the second or third leaves from each ear or corn tassel) were used for extraction. The number of aphids on the leaves were counted before the leaves were homogenized with 60 ml of distilled water. The homogenates were filtered through cheese cloth, and the filtrates were adjusted to pH 3.0 by adding 1.0 N HCl. They were centrifuged at $10,000 \times g$ for 10 min at 2°C using a Hitachi 18PR-52 centrifuge. The supernatant was collected and extracted 3 times with an equal volume of ethylether. The ethylether fractions were concentrated in vacuo and the concentrate was subjected to liquid chromatography and GC-MS analysis.

Liquid chromatography. Aconitic acid was determined by a Shimadzu HPLC 5A with a SPD-2A detector, a Hibar® column with precolumn (Cica-Merck), and a Shimadzu C-6RA recorder. For elution we used a mixture of 0.05 M phosphate buffer (pH 3.5) and acetonitrile (80:20 v/v). The flow rate was adjusted to 1 ml/min. Components were detected at 250 nm.

Chemicals. 99+ % (Ti) grade of (*E*)-aconitic acid and (*Z*)-aconitic anhydride were purchased from Wako Pure Chemical Ind.

GC-MS analysis. Aconitic acid was identified as methyl aconitate by GC-MS, after being esterified by adding BF₃-Methanol. Two ml of BF₃-Methanol was added to a part of the ethylether solution. After incubation for 10 min at 60°C, 10 ml of *n*-hexane and 15 ml saturated NaCl-solution were added. The *n*-hexane fraction was subsequently used for GC-MS analysis. Mass spectra were obtained on a Shimadzu QP1000(A) combined with a GC-15A gas chromatograph (glass column, 2 mm × 2 m). Peaks, which were resolved on a 10% FON column with a temperature program of 70°C to 240°C at 8°C/min and with a He flow of 40 ml/min, were scanned at 70 eV from *m/z* 35 to 300 (ion source 250°C). The ethylether extracts of each plant, including other unknown substances, were esterified in the same manner and used for identification.

Artificial rearing and toxicity assays of aconitic acid. Artificial diets for *Ropalosiphum padi* (KIECKHEFER and DERR, 1967) and for *Schizaphis graminum* (CRESS and CHADA, 1971) were prepared and assayed by the parafilm method (KANEHISA et al., 1990). Usually, 3 aphids per bottle (mostly second instar nymphs) were used. The effects of (*E*)-aconitic acid and (*Z*)-aconitic acid on survival rates were determined. These rates were compared with those rearing on distilled water only.

RESULTS

Separation of aconitic acid from cereal plant extracts by liquid chromatography

Acid-soluble fractions from plant extracts were further separated by liquid chromatography as shown in Fig. 1. The peaks of (*E*)-aconitic acid and (*Z*)-aconitic acid appeared about 3.8 min after injection, and (*E*)- and (*Z*)-forms could not be separated. More than 10 other peaks were detected in the plant extracts, with especially large peaks in corn. Although chromatographic profiles differed between species and growth stage, aconitic acid was a prevailing component in wheat, corn, sorghum, barnyard grass and rice. Inconsistent and rather small amounts were found in barley.

Fig. 1. Elution profiles of acid-soluble extracts, A: wheat, B: barley, C: corn and D: sorghum, a=aconitic acid.

Fig. 2. Selected ion-monitoring chromatograms of methylester of (A): (*E*)-aconitic acid, B: corn (W-15 line, July 3), C: wheat (Norin-54 line, May 7) and D: barley (Aizu 4+Aizu 6 lines, Apr. 19).

Identification of aconitic acid by GC-MS

The monitoring chromatograms in GC-MS analysis are shown in Fig. 2. The peaks of methylesters of authentic (*E*)-form and (*Z*)-form appeared 20.2 min and 20.1 min after injection, respectively. The electron-impact mass spectrum of methylester of authentic (*E*)-aconitic acid showed diagnostic ion peaks at m/z 59 (100%, COOMe), 216 (M^+ , weak), 185 (41%, M-OCH₃), 184 (63%, M-CH₃OH), and 157 (31%, M-CO₂CH₃). Other peaks of 156 (70%), 153 (99%), 125 (43%), 113 (25%), and 98 (24%) were observed in the authentic sample. These peaks were also used for the identification of the plant extracts. The methylester of (*Z*)-aconitic acid showed almost the same mass spectrum. The methylesters of (*E*)-form and (*Z*)-form could not be separated in the plant samples, so we calculated aconitic acid in its entirety. Small amounts of aconitic acid in barley lines were also revealed by the monitoring chromatogram.

Contents of aconitic acid in cereal plants, and densities of aphid populations

Aphids appeared on wheat and barley in early April, with the first population

Table 1. Aconitic acid content ($\mu\text{g/g}$ fresh weight) of wheat leaves and (in parentheses) densities of aphid population (number of attached aphids/5 g=number of leaves)

Line (Stage)	Mar. 28 (50 cm)	Apr. 11 (80 cm)	Apr. 24 (booting)	May 7 (e. dev.)	May 21 (mat. sen.)
Norin-1	20.2 (0=14)	15.6 (3=11)	17.0 (10=12)	18.6 (6= 9)	11.4 (37= 8)
Norin-54	24.4 (0=27)	18.6 (0=20)	19.0 (4=11)	18.0 (4= 8)	7.2 (24=10)
Norin-56	18.6 (0=22)	19.0 (5=21)	17.6 (7=23)	14.8 (0=10)	10.8 (24=12)
Norin-61	16.6 (0=20)	20.2 (0=20)	16.6 (3=18)	13.6 (6=19)	5.8 (26=19)
Shiro*	13.2 (0=19)	17.4 (5=22)	23.6 (5=19)	11.4 (11=11)	5.4 (16=12)
Chinko-1	21.4 (0=21)	24.2 (0=16)	23.6 (3=15)	12.2 (18= 8)	6.6 (14=10)
Shirasagi	12.8 (0= 8)	21.4 (1=10)	22.6 (5= 9)	9.2 (13= 7)	8.4 (13=12)
Nakasoushu	14.0 (0=18)	23.2 (0=16)	21.8 (4=12)	16.8 (15=10)	9.2 (7=10)

*: Shiroemidashi, (mat. sen.): mature or senescent, (e. dev.): ear development

Table 2. Aconitic acid content ($\mu\text{g/g}$ fresh weight) of barley leaves and (in parentheses) densities of aphid population (number of attached aphids/5 g=number of leaves)

Line (Stage)	Feb. 19 (20 cm)	Apr. 9 (60-90 cm)	Apr. 19 (booting)	Apr. 27 (e. e. dev.)	May 28 (mat. sen.)
Gose-hen** (S)	3.0 (0=24)	7.6 (0=15)	5.3 (18= 8)	(115= 7)	9.0 (14= 8)
f9-g13-Hsk (S)	2.6 (0=26)	6.8 (39=12)	5.3 (106=10)	(126=10)	9.3 (25=14)
Kirin* (S)	4.1 (0=30)	8.6 (9=10)	3.2 (9= 8)	(60=10)	3.2 (17= 8)
Aizu-4 (S)	3.4 (0=41)	4.5 (2=13)	6.2 (22= 7)	(15=11)	4.4 (15=14)
Aizu-6 (S)	5.1 (0=46)	6.2 (1= 8)	4.6 (11=10)	(32= 9)	9.5 (25=13)
Nihon-iochi (R)	2.7 (0=29)	5.3 (0=10)	+ (3=12)	(5= 9)	+ (14= 7)
Kikai*** (R)	6.3 (0=28)	+ (0=10)	+ (7=12)	(0=13)	+ (8= 9)
Koike**** (R)	2.7 (0=25)	2.3 (0= 9)	+ (2= 9)	(0= 8)	9.8 (17= 8)
H. sp. 2558 (R)	4.0 (0=32)	2.6 (0=13)	6.8 (0=21)	(2=14)	+ (0=23)
H. sp. 4969 (R)	3.4 (0=34)	6.1 (1=20)	5.7 (2=18)	(0=15)	4.5 (4=20)

*: Gosheshikokoku-1-hen, **: Kirin-tyoku-1, ***: Kikai-hadaka, ****: Koike-Rokkaka,
+: inconsistent and low amount, (e. e. dev.: early ear development), (S): susceptible
line, (R): resistant line.

Table 3. Aconitic acid content ($\mu\text{g/g}$ fresh weight) of corn leaves and (in parentheses) densities of aphid population (number of attached aphids/plant)

Line (Stage)	June 11 (80 cm)	July 3 (e. e. dev.)	July 17 (2nd time) (l. e. dev.)	July 21 (3rd time) (mature)	July 27 (mat. sen.)
A-171	316 (0)	235 (0)	(68)	(7)	180 (0)
A-554	304 (0)	290 (0)	(93)	(396)	225 (0)
A-632Ht	424 (0)	225 (0)	(201)	(237)	175 (0)
D-321	260 (0)	250 (0)	(134)	(10)	225 (0)
H0-11	224 (0)	400 (0)	(721)	(241)	250 (0)
A-661	568 (0)	435 (0)	(4)	(10)	445 (0)
CM-51	416 (0)	230 (0)	(25)	(21)	510 (0)
N-150	272 (0)	520 (0)	(8)	(25)	315 (0)
SD-10	384 (0)	470 (0)	(5)	(10)	460 (0)
W-15	300 (0)	470 (0)	(3)	(4)	410 (0)

Aphids did not appear at the experimental field until ear-development time, so *Rhopalosiphum maidis* was transferred from other infested fields (for the first time on June 21), but most aphids disappeared within a week. The second transfer was on July 11 and the aphids were counted on July 17. The third transfer was on July 17 and the count was done on July 21. Almost all aphids were dead at the end of July, maturing time. (e. e. dev.): early ear development, (l. e. dev.): late ear development.

Table 4. Aconitic acid content ($\mu\text{g/g}$ fresh weight) of sorghum leaves and (in parentheses) population densities of *Melanaphis sacchari* (number of attached aphids/10 g of leaves)

Line (Stage)	July 11 (50–70 cm)	July 24 (e. e. dev.)	August 12 (e. dev.)	August 29 (e. dev.)
Redlan-B (S)	115 (0)	95 (692)	135 (692)	215 (1260)
PE-954177 (R)	380 (0)	175 (0)	435 (20)	675 (0)

(S): susceptible line, (R): resistant line, (e. e. dev.): early ear development, (e. dev.): ear development.

Table 5. Aconitic acid content ($\mu\text{g/g}$ fresh weight) in barnyard grasses and rice

Plant (Stage)	Aug. 6 (80–100 cm)	Aug. 30 (booting)	Sept. 17 (e. e. dev.)	Oct. 9 (e. dev.)
<i>Echinochloa oryzicola</i> VASING	160	420	540	580
<i>E. crus-galli</i> BEAUV.	200	310	110	85
<i>E. crus-galli</i> var. <i>formosensis</i> OHWI	260	100	95	70
<i>Oryza sativa</i> L. (Akebono)	310	230	130	100

(e. e. dev.): early ear development, (e. dev.): ear development.

peak occurring from the end of April to early May. Their second peak was in the end of May in senescent plants. The contents of aconitic acid of wheat lines are shown in Table 1. All wheat lines showed more resistance to aphids than susceptible barley lines. The aphid colonies were observed at different places throughout the observation period, usually once a week, and did not appear to be stable. Table 2 shows that the amounts of aconitic acid in wheat were several times higher than those in barley and about 10 times lower than those in corn and in sorghum.

Table 6. Survival percentage of *Rhopalosiphum padi* and *Schizaphis graminum* on diets with different concentrations of (*E*)-aconitic acid and (*Z*)-aconitic acid

Treatment	Total number of aphids (aphids× trials)	Days															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>Rhopalosiphum padi</i>																	
Control	36 (3×12)	100	95	85	81	75	60	46	29	14	8						
H ₂ O	24 (3× 8)	100	83	58	25	8	0										
(E)-acon.a.1.0 %	12 (3× 4)	92	42	0													
"	0.3 %	83	75	24	4	4	0										
"	0.1 %	89	77	69	52	43	29	6	4	2	0						
"	0.03%	100	96	92	71	42	21	13	13	8	0						
"	0.01%	100	100	92	75	50	29	17	8	0							
(Z)-acon.a.1.0 %	24 (3× 8)	96	67	29	4	0											
"	0.3 %	92	75	36	4	4	0										
"	0.1 %	100	80	46	21	21	17	17	0								
"	0.03%	100	80	58	38	33	24	24	8	0							
"	0.01%	100	96	67	46	33	24	12	4	4	0						
<i>Schizaphis graminum</i>																	
Control	36 (3×12)	100	100	100	92	80	69	69	69	58	50	50	47	33	25	14	3
H ₂ O	36 (3×12)	100	97	78	28	22	6	0									
(E)-acon.a.0.3 %	24 (3× 8)	100	100	83	63	38	30	25	16	16	8	8	0				
"	0.1 %	100	96	88	67	67	67	62	33	29	21	8	4	0			
"	0.03%	100	100	96	79	75	71	67	62	50	33	29	8	8	4	0	
"	0.01%	100	100	96	83	75	75	75	62	50	33	29	21	12	4	0	
(Z)-acon.a.0.3 %	24 (3× 8)	100	100	71	50	37	33	29	21	16	4	0					
"	0.1 %	100	100	87	87	71	71	54	50	46	29	21	8	0			
"	0.03%	100	100	92	92	79	71	54	50	29	12	12	12	8	8	4	0
"	0.01%	100	100	96	87	83	67	62	50	46	33	25	21	16	8	8	0

The corn lines contained very high amounts of aconitic acid (Table 3). In 1989 and 1990, aphids did not appear on our experimental lines, so *R. maidis* were collected from other severely infested corn fields and transferred to the experimental plots 3 times. Their densities decreased rapidly within a week.

A clear difference between susceptible and resistant lines was observed in sorghum (Table 4). Aconitic acid was more prevalent in the resistant line than in the susceptible one.

The contents of aconitic acid in barnyard grasses and of a rice line are shown in Table 5. No population of aphids was found during the experimental period. The level of aconitic acid in *E. oryzicola* corresponded to plant age.

Effect of aconitic acid on the mortality of aphids fed on artificial diets

The results are shown in Table 6. *S. graminum* could survive longer than *R. padi* on each of the artificial rearing solutions. (*E*)- and (*Z*)-aconitic acids had similar effects. At concentrations higher than 0.3%, (*E*)- and (*Z*)-aconitic acids acted like antibiotic substances, inducing higher mortality than distilled water. Survival rates of control and test groups differed at concentrations higher than 0.01%. *S. graminum* was less sensitive to aconitic acid than *R. padi*.

DISCUSSION

Aconitic acid has been found in many plants such as Gramineae and Ranunculaceae, comprising up to 6% of the dry weight. It has been assumed that most of the aconitate is present in the (*E*) form (BURAU et al., 1965; STOUT et al., 1967; CLARK, 1972; PATTERSON et al., 1972; KIM et al., 1976; THOMPSON et al., 1988). The (*Z*) form is known as an important substance in the KREBS cycle of cellular metabolism, while the (*E*) form is not a substrate for the aconitate enzyme and cannot directly enter the KREBS cycle (MACLENNAN and BEEVERS, 1964; PATTERSON et al., 1972). The (*E*) form caused accumulation of citrate while lowering respiratory rates in slices of rat kidney cortex (SAFFRAN and PRADO, 1949). Although we could not discriminate between the (*E*)-form and the (*Z*)-form by liquid and gas chromatographies in this experiment, both forms had similar effects in the rearing experiments, showing that these acids may directly effect the digestive organ.

All corn lines and the resistant sorghum line contained high amounts of aconitic acid (200 to 600 µg/g of fresh leaf), had almost no aphid populations. The susceptible line of sorghum which contained a low amount of aconitic acid had a high aphid density. In sorghum, duhrin alkaloid and 2 other substances have been reported to impart plant resistance (DRYER et al., 1981). However, higher amounts of aconitic acid may also play a role in resistance.

Wheat lines containing moderate amounts of aconitic acid showed rather weak resistance properties. Barnyard grasses contained high amounts of aconitic acid. Accumulation of aconitic acid in the maturing stage of greenish barnyard grass (*E. oryzicola*) is probably related to the prevalence of healthy plants in brownish hopper-infested paddy fields, where (*E*)-aconitic acid may deter feeding (KIM et al., 1976). Two other subspecies and a rice line showed gradual decreases in aconitic acid with progressive stages.

Barley has small and inconsistent amounts of aconitic acids, with resistance caused

by a gramine alkaloid (CORCUERA, 1984; KANEHISA et al., 1990). The occurrence of gramine is characteristic for barley lines. No gramine has been found in wheat, corn, sorghum, barnyard grass or rice lines (unpublished observations).

Rearing on aconitic acid concentrations higher than 0.01% caused higher mortality than the artificial diet. KAWADA and LOHAR (1989) have observed by the electronic monitoring method that gramine added to artificial diets significantly reduced the duration of ingestion and increased the number of probes and duration of salivation.

Aconitic acid showed an effect about 10 to 100 times weaker than the gramine alkaloid (KANEHISA et al., 1990), the effect on *S. graminum* was especially weak.

Each plant has unique metabolic patterns and is assumed to have unique phloem sap. The aphid is known to feed on phloem sap. Localization of aconitic acid and its concentration in phloem sap are important characteristics in sucking insects, but studies have been rare. Nevertheless, it is now clear that high amounts of aconitic acid display resistance activity to cereal aphids.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Dr. O. SAITO of Hokkaido National Agricultural Experimental Station for providing corn seeds, to the staff of Chugoku National Agricultural Experimental Station for providing sorghum seeds, to Drs. S. YASUDA and T. KONISHI of our institute for providing wheat seeds and barley seeds, and to Dr. K. NISHI of our institute for providing barnyard grasses and rice. We also wish to thank Dr. Harm HUTTINGA, Department for Detection and Biotechnology of the Research Institute for Plant Protection, Wageningen, Netherland, and Guest Professor of the Institute for Bioresources, Okayama University, for critically reading the manuscript.

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