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Further Observations on the Relationship between Aconitic Acid Contents and Aphid Densities on Some Cereal Plants

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INTRODUCTION

Plant-feeding arthropods, in the course of evolution have adapted differentially to their plant host and thereby gained access to the available resource. Plants responded by developing chemicals and morphological defense to arthropods. Some secondary metabolic substances in plants have been assumed to be toxic or deter feeding. Aconitic acid in the barnyard-grass subspecies, *Echinochloa oryzycola* Vasing has been reported to be an active anti-feeding component against brown rice planthopper *Nilapalvata lugens* (Stål) (Kim et al. 1976). Aphids, *Ropalosiphum padi* L., *R. maidis* (F.), *Schizaphis graminum* (Rondani) and *Sitobion akeviae* (Shinji), are important pests of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.). On barley plants, more than seven aphids per stem causes yield loss (Hansen 1990). *R. maidis* sometimes severely infested corn plants (*Zea mays* L.), the leaves being wet by honeydew. *Melanaphis sacchari* (Zehntner) caused brownish decay of leaves of a susceptible line of sorghum *Sorghum bicolor* Moench. We examined the relation between aconitic acid contents and aphid densities in wheat, barley, corn, sorghum, barnyard grass and rice (Rustamani et al. 1992a). The plants containing larger amounts of aconitic acid showed a moderate degree of resistance to aphids. The findings obtained on additional lines in 1991, and on other components, such as DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), are reported herein.

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MATERIALS AND METHODS

Cereal Plants

Sixteen lines of corn (Table 1) were obtained from Hokkaido Agricultural Experimental Station. Four to seven plants per line were cultured. They were sown at the end of April and harvested in August. Resistant and susceptible lines of sorghum (Table 2) were obtained from Chūgoku Agricultural Experimental Station. Ten plants were cultured per line. They were sown in early May and harvested in November. Three subspecies of barnyard grass, *Echinochloa oryzycola* Vasing, *E. crus-galli* Beauv. and *E. crus-galli* var. *formosensis* Ohwi and two lines of rice, *Oryza sativa* L. (Table 3) were obtained from the laboratory of Environment and Ecological Adaptation of our Institute. They were cultured at a rice field by normal cultivation and sampled. Nine lines of barley (Table 4) were obtained from the Barley Germplasm Center of our Institute. Eight lines of wheat (Table 5) were obtained from the Laboratory of Plant Genetics of our Institute, they were sown in mid-November and harvested early in June. About 200 tillers of each line of barley and wheat were cultured.

Aphid densities

The number of aphids attached to each plant was counted per stem and compared among the lines. From their appearance until harvest time, the aphids on barley and wheat were counted once a week and those on other plants once every two weeks. *R. maidis* was introduced from other severely infested fields to the experimental corn field, because almost all lines showed no stational aphid colony. Almost no aphids were observed on barnyard grasses and rice lines, but *M. sacchari* was observed once on *E. crus-galli* plants.

Extraction and Estimation of Aconitic Acid

Five or ten g fresh weight leaves, usually the second or third leaf from each ear or corn tassel were used for extraction. The aphids on the leaves were counted, and the leaves were homogenized with 80 ml of distilled water. The homogenate was filtered through cheese cloth, and the filtrate adjusted to pH 3.0 by addition of 1.0 N HCl. The filtrate was centrifuged at 10,000x*g* for 10 min at 2℃ using a Hitachi 18PR-52 centrifuge. The supernatant was collected and extracted with ethylether. The ethylether fractions were concentrated in vacuo and the concentrate was subjected to liquid chromatography.

Chemicals and Identification

Pure 99+% grade of (*E*)-aconitic acid was purchased from Wako Pure Chemical Industry. Aconitic acid in the plant extract was identified by GCMS, using a Shimadzu QP1000 (A) combined with a GC-15A gas chromatograph, after making methylester by treatment of BF_3-MeOH (Rustamani et al. 1992b). Pure DIMBOA was obtained from Dr. O. Saito, Hokkaido Agricultural Station. DIMBOA in plant extract was identified by the brown spot produced using a ferric chloride reagent {50g $FeCl_3 \cdot 6H_2O$, 500 ml EtOH, 5 ml HCl (11.5 M)} on silica gel (Merck 60G) thin layer chromatography, developed with MeOH : $CHCl_3$: acetic acid (50 : 50 : 1, v/v).

High Performance Liquid Chromatography (HPLC)

Aconitic acid and DIMBOA were quantitatively determined by a Shimadzu HPLC 5A with a SPD-2A detector, a Hibar[R] column with precolumn (Cica-Merck), and a Shimadzu C-6RA recorder. For elution we used a mixture of 0.05M 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. Amounts were calculated from the profile for the authentic compounds.

RESULTS AND DISCUSSION

Acid soluble components in cereal plants

More than twenty components were observed as high and low peaks in the HPLC profile which are shown in Fig. 1. They varied with the plant species, lines and ages. Some of them are the same compounds, but many are different and unidentified compounds. Aconitic acid was the preceding component in all cereal plants as reported (Rustamani et al. 1992a). DIMBOA is known as a resistant component in corn to the European corn borer, *Ostrinia nubilalis* (Hubner), (Klun et al. 1967) and in wheat to the cereal aphids (Argandona et al. 1980, Corcuera et al. 1982). The presence of DIMBOA in corn was confirmed, but obscure in other cereal plants.

Corn

The corn lines contained larger amounts of aconitic acid (Table 1) than the other cereal plants. The amounts gradually decreased with age. Very few aphids, mostly *R. maidis*, appeared occasionally in experimental plots, but no stable colony was observed as in 1989 and 1990 (Rustamani et al. 1992a). Therefore *R. maidis* was collected from other severely infested corn fields and transferred 2 times to 4 plants of the each line in the experimental plots. They were counted on plants three

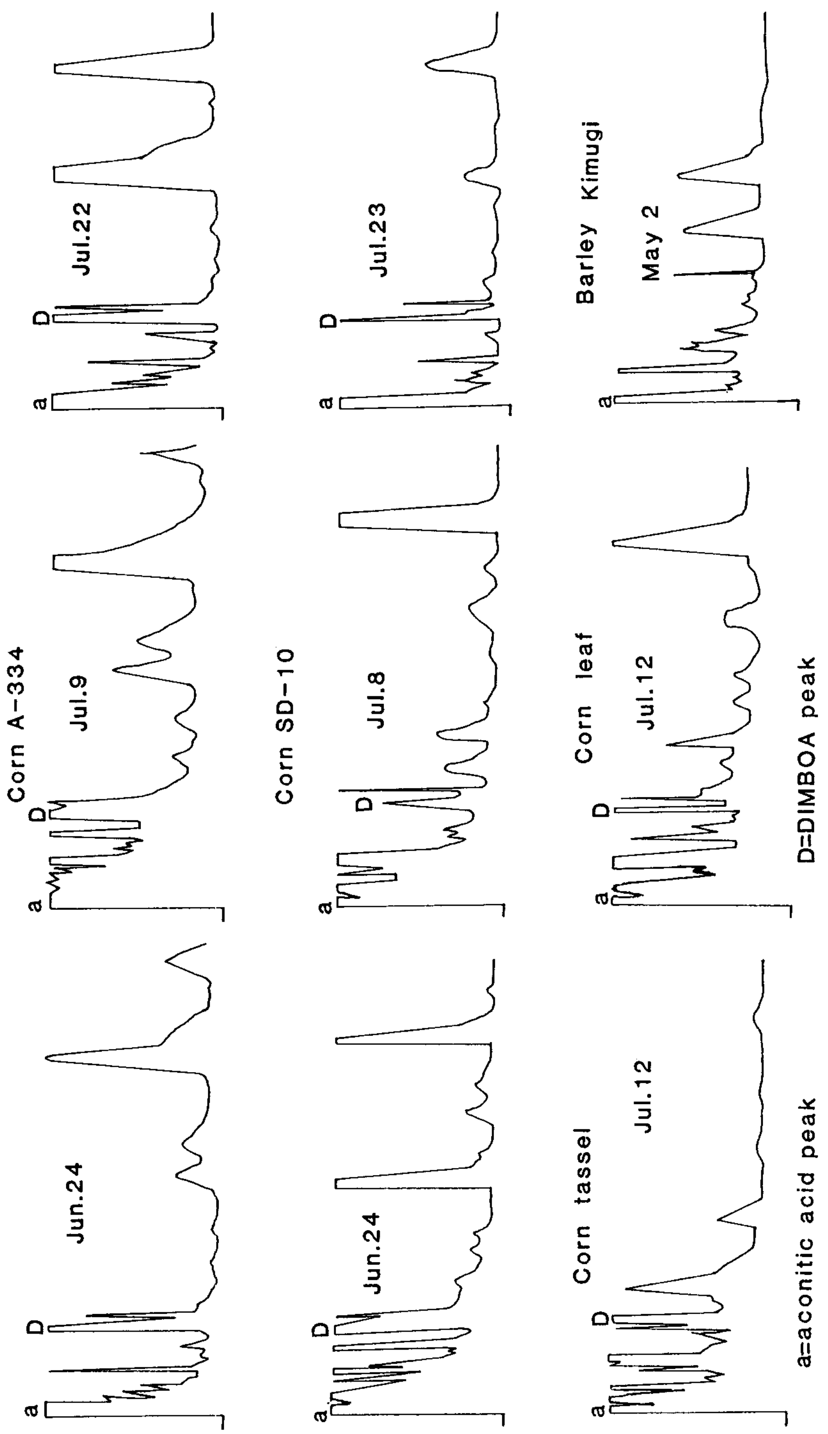

Fig 1-a. HPCL profiles of acid soluble components of cereal plants

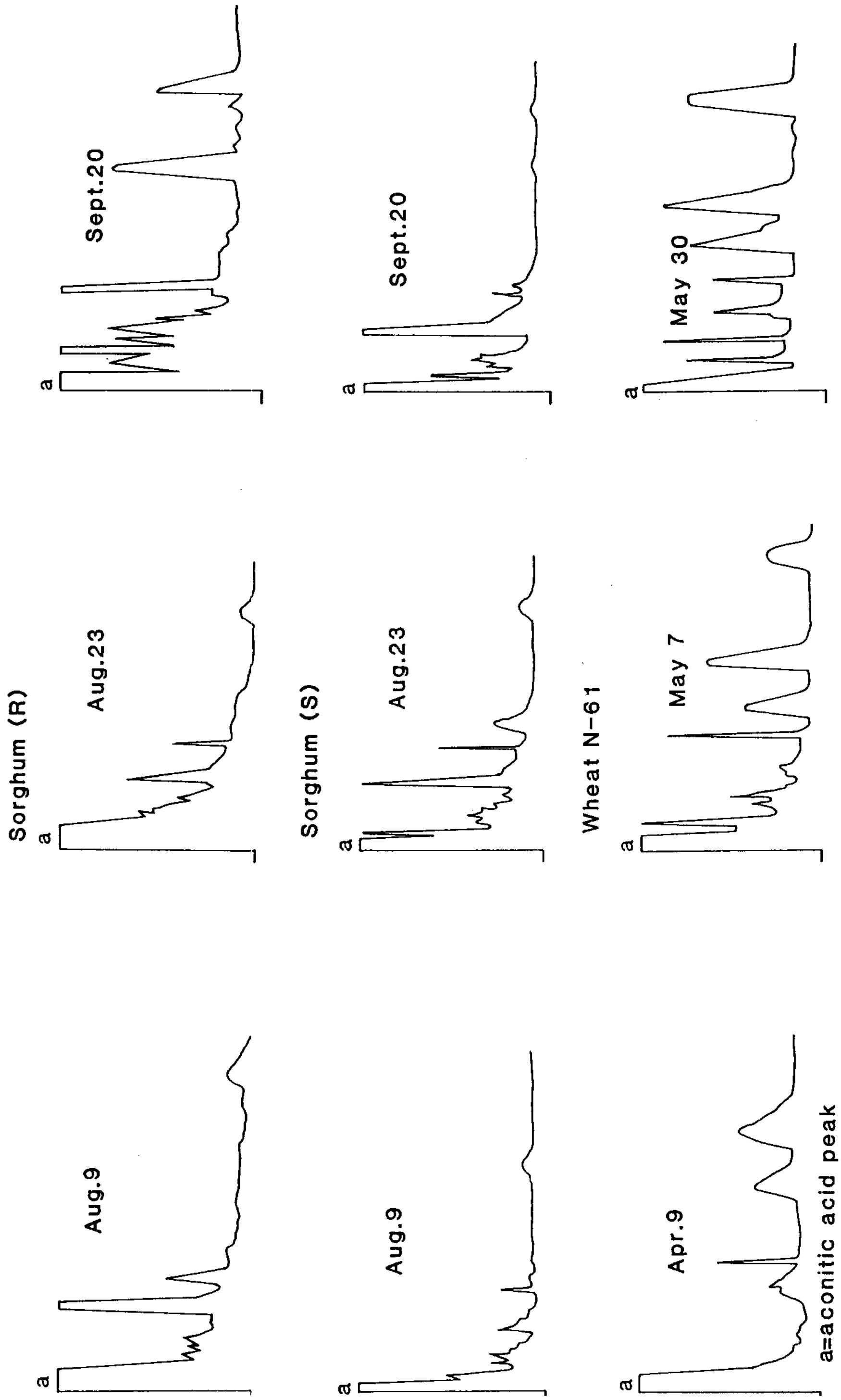

Fig 1-b. HPCL profiles of acid soluble components of cereal plants

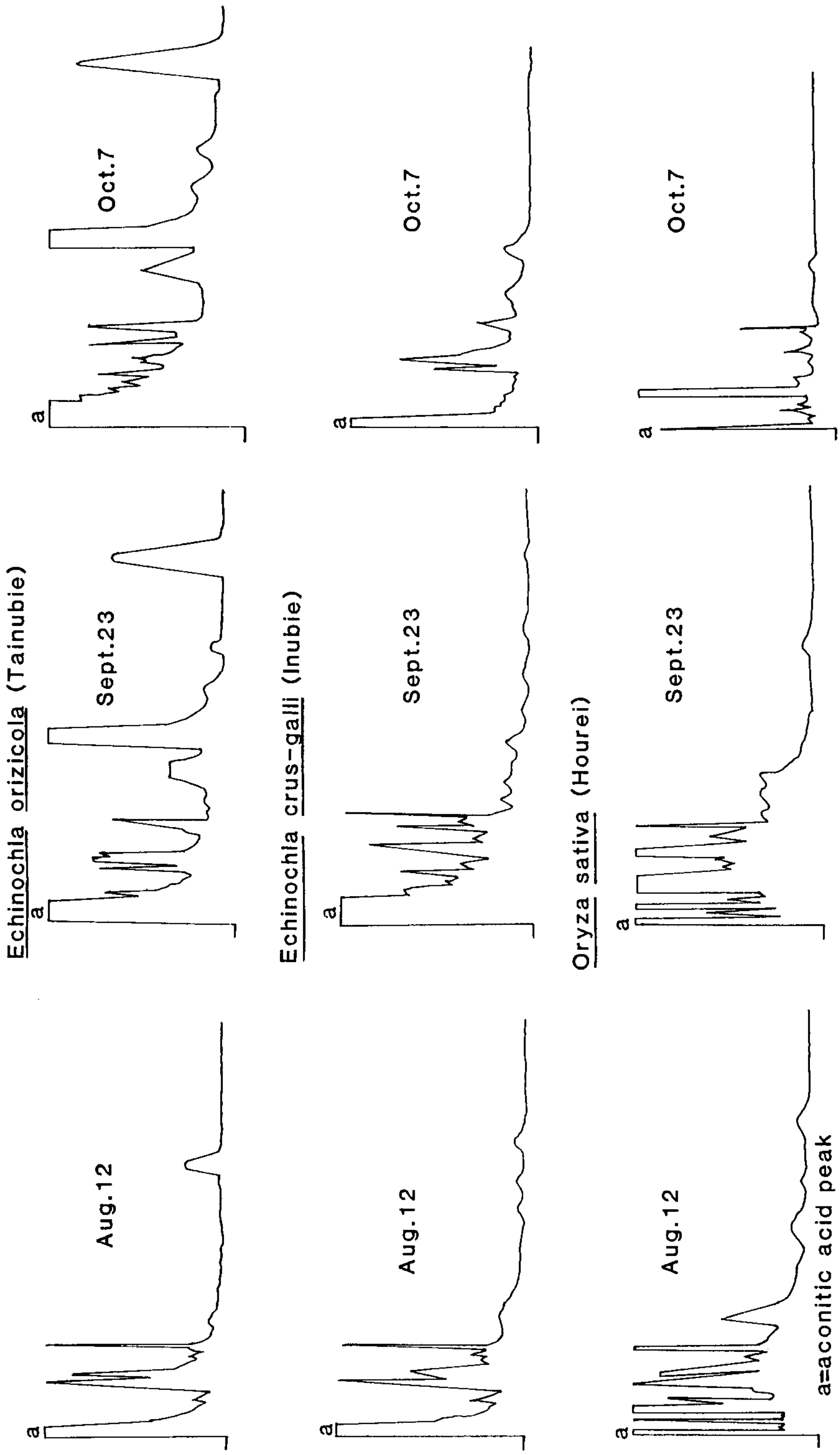

Fig 1-c. HPCL profiles of acid soluble components of cereal plants

Table 1. Aconitic acid contents ($\mu g/g$ w.w.) of corn leaves in 1991

Line		June 21* aphids	June 24	July 2* aphids	July 6* aphids	July 8	July22
A-334		0	495(0)	0	0	309(0)	247(0)
A-619Ht	+	0	300(0)	45	0	247(10)	207(0)
A-654Ht		14	315(0)	0	0	333(0)	201(0)
A- 34	+	12	357(1)	2	0	251(0)	135(0)
N-132		3	442(0)	2	0	321(0)	320(0)
N-150		0	360(0)	2	0	387(0)	158(0)
Ho-6		0	344(0)	0	0	291(0)	240(0)
D-321		0	302(0)	10	0	251(0)	155(0)
A-632Ht	+	26	285(3)	940	103	270(5)	184(14)
A-661	+	20	310(0)	140	76	208(11)	134(20)
CM-51	+	48	228(2)	270	90	70(38)	50(51)
CO-108	+	230	210(8)	276	40	216(23)	180(31)
W-540	+	36	218(7)	47	60	112(23)	50(40)
W-729D	+	300	295(31)	2307	22	136(38)	127(20)
ND-100	+	6	244(6)	832	113	158(15)	130(13)
SD-10	+	23	172(15)	2115	1004	200(23)	155(51)

* : *R. maidis* was transferred twice on June 12 and June 28, and all aphids on 4 plants of the same line counted. + : infested leaf was used for assay.
Numbers in parentheses show the number of aphids on leaves of a 10 g sample.

times, i.e. June 21, July 2 and 8, respectively. In parentheses are given the number of aphids on the leaves of the 10 g sample used for extraction. Aphids attached leaves were intentionally sampled, though there were only a few such leaves on the same plant, because a difference was expected between aphid-attached leaves and non-attached leaves. There were two different groups, in one group which had a larger amount of aconitic acid, the aphid density decreased rapidly and seemed to have a higher resistant property. A-334 (Fig.1) is an example. In the other group, the aphid density decreased slowly, and the amount of aconitic acid was smaller than that in the first group and seemed to have a lower resistant property. SD-10 (Fig.1) is an example. Severely infested tassel and leaves of unknown lines were collected from a farm. They also had smaller amounts of aconitic acid. This was a new finding made in 1991. The larger amount of aconitic acid may play a role in resistance to aphid, although DIMBOA and some other unknown substances may also share a role in the resistance property.

Sorghum

A clear difference was observed between susceptible and resistant lines of sorghum (Fig.1 and Table 2). The amount of aconitic acid in the resistant line was two times larger than that in the susceptible line.

Table 2. Aconitic acid contents (μg/g w.w.) of sorghum leaves in 1991

Line	Aug. 9	Aug. 23	Sept. 6	Sept. 20
Redlan-B(S)	255(320)	230(328)	245(64)	160(513)
PE-954177(R)	510(0)	495(8)	425(12)	420(5)

Numbers in parentheses show the number of aphids on 10 g sample.

Barnyard grass and Rice

These plants are rarely attacked by aphids. Once a few *M. sacchari* were observed on *E. crus-galli. E. crus galli* var. *oryzycola* had larger amounts of aconitic acid than the other barnyard grasses and rice lines, especially large amounts in the maturing stage of the plant (Fig. 1 and Table 3). The mature plant of this subspecies has been reported as a healthy green plant in a rice field infested with brown planthoppers. Its resistance to planthopper is a serious problem in rice culture. Rice contained smaller amounts of aconitic acid and the amount decreased markedly with age. However, the rice lines showed other major peaks, the activities of which are obscure (Fig.1).

Table 3. Aconitic acid contents (μg/g w.w.) of barnyard grasses and rice plants in 1991

Plant	Aug. 12	Sept. 2	Sept. 23	Oct. 7
Echinochla oryzycola	208	262	350	410
E. crus-galli	460	272	144	145
E. crus-galli var. *formosensis*	315	220	115	90
Oryza sativa (Akebono)	355	342	224	110
O. s. (Hourei)	105	62	65	30

Table 4. Aconitic acid contents (μg/g w.w.) of barley leaves in 1991

Line	Apr. 4	Apr. 18	May 1	May 15
K-gl_3-Bl	40.0(0)	4.0(36)	5.8(46)	1.1(36)
Kimugi	30.0(0)	13.0(0)	27.0(17)	20.0(62)
Smyrna	38.0(0)	12.0(8)	17.0(4)	4.4(7)
Munshinchiang-1	13.8(7)	13.2(7)	12.0(1)	7.8(4)
Amaki-2	22.0(0)	13.2(0)	10.4(6)	8.2(6)
H. s. MRC-4	25.6(0)	22.6(0)	13.2(0)	4.6(0)
H. s. MRC-6	22.0(0)	16.0(0)	11.6(8)	10.4(2)
H. sp. 4969	10.8(10)	8.8(16)	8.2(17)	3.6(22)
H. s. 15(b)5024	64.0(0)	38.0(8)	12.2(38)	9.8(2)

Numbers in parentheses show the number of aphids on leaves of a 10 g sample.

Barley

The amount of aconitic acid in barley was smaller than that in the above cereals and gradually decreased with age (Table 4). Aphids appeared on barley in early April, and the first population peak was from the end of April to early May. The second peak was from the end of May to early June on the senescent plants. Our institute has typical resistant lines and susceptible lines to aphids. The importance of gramine, 3-N,N-dimethylaminomethylindole, in barley resistance to aphids was reported (Kanehisa et al. 1990, ; Rustamani et al. 1992b). Gramine was found only in barley, and not in any of the other cereals tested in this experiment. In barley aconitic acid may not be important for the resistance property to aphids, because of its amount was small.

Table 5. Aconitic acid contents (ug/g w.w.) of wheat leaves in 1991

Line	Apr. 8	Apr. 22	May 7	May 20	May 30
Norin-54	59.0(0)	38.2(14)	27.0(23)	4.8(30)	2.6(28)
Norin-56	67.0(2)	30.0(6)	23.0(8)	16.0(10)	8.5(24)
Norin-61	72.2(0)	59.4(2)	24.0(6)	4.0(11)	3.6(36)
Shiroemidashi	34.5(0)	24.0(17)	13.0(18)	2.0(23)	1.6(81)
Chinko-1	53.5(8)	35.6(16)	30.0(12)	7.2(36)	5.0(62)
Mubochinko	75.0(0)	40.0(28)	29.0(32)	9.9(18)	4.6(89)
Shirasagi	29.4(0)	21.6(26)	17.0(28)	15.2(39)	5.2(96)
Nakasoushukinai	30.6(5)	22.4(14)	21.6(19)	5.2(39)	3.0(98)

Numbers in parentheses show the number of aphids on leaves of a 10 g sample.

Wheat

The amounts of aconitic acid in wheat was similar to that in barley and a tendency to decrease with age was observed (Table 5). We could not obtain any typical wheat lines resistant or susceptible to aphids, all tested lines had a weak resistant property in comparison with very susceptible barley lines. DIMBOA was not identified in wheat by HPLC. The resistance factor in wheat is unknown.

Aconitic acid has been found in many plants, such as Graminae and Ranunculacae (Burau et al. 1965 ; Stout et al. 1967 ; Clark 1972 ; Patterson et al. 1972 ; Kim et al. 1976 ; Thompson et al. 1988 ; Rustamani et al. 1992a). Aphids are phloem sap feeders. (*Z*)-Aconitic acid is known as a substrate in the Krebs cycle of the cellular metabolism. However, we could not distinguish the (*E*)-form from the (*Z*)-form or the concentration in phloem sap. Resistant corn and sorghum lines contained more than 200 μg/g wet weight leaves. Susceptible lines had less than 200 μg/g. Previously, we reported that aconitic acid had a 10 to 100 times weaker effect than the gramine in the survival percents on a rearing solution, especially weak activity to *S. graminum* in comparison with *R. padi* (Rustamani et al. 1992a).

Cereal plants contained unidentified components as big or middle size peaks (Fig.1). These peaks changed with plant growth and we cannot deny the importance of these unknown substances. There are many antifeedants in plant tissues, but they are taking path ways of mutual coevolution between plants and feeding animals. Trace amounts of a few phenolic acids in cereal plants were identified by GC-MS, and they had anti-feeding activity in a rearing experiment (unpublished observation). The resistance of cereals to aphids may be controlled by many substances. However, aconitic acid was a preceding larger component in some cereals, in them it may play an important role.

SUMMARY

The relationship between aconitic acid contents and resistance property to aphids in cereal plants was investigated in 1991, and compared with previous reports. Sixteen corn lines, 2 sorghum lines, 3 subspecies of barnyard grasses, 2 rice lines, 9 barley lines and 8 wheat lines were compared.

Plant lines which have more than 200 μg aconitic acid per g wet weight leaves of corn and resistant sorghum showed resistance to aphids in comparison with those with a smaller amount. *E. crus-galli* var. *oryzycola* had very large amounts of aconitic acid in the mature stage, and were different from other barnyard grasses and rice. Barley and wheat contained less than 60 μg/g, an amount insufficient to show a resistant property, and play a role of as the gramine in barley. Aconitic acid was considered as a moderate anti-feedant to aphids.

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Relationship between Aconitic Acid Contents and Aphid Densities on Some Cereal Plants

数種禾穀類におけるアコニット酸の含量とアブラムシ密度との関係

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要 約

禾本科植物の葉に含まれているアコニット酸を抽出定量し、寄生性のアブラムシに対する耐性との関係を調べた。トウモロコシの16系統、典型的な抵抗性と感受性のソルガムの2系統、ヒエ類の3亜種、イネの2系統、オオムギの9系統とコムギの8系統について比較した。

一般的に若齢期の植物は老熟期の植物より高い含量であった。トウモロコシは全体として200μg/g以上を含有する系統が多く、アブラムシに対して耐性を示した。外部からトウモロコシアブラを導入し、その推移とアコニット酸の関係を調べたところ、比較的耐性を示す系統は多量に含有し、アブラムシが寄生し易い系統は少量であった。

ヒエノアブラムシに耐性のソルガムの系統は感受性の系統の約2倍のアコニット酵を含有していた。タイヌビエのみがイヌビエ、ヒメタイヌビエ、イネのホウレイとアケボノと異なって、成熟するにつれてアコニット酸の含有量が増加していた。このことはトビイロウンカによって稲田が坪枯れの時にタイヌビエのみが青青としており、それはアコニット酸によるとの報告と一致している。

オオムギは比較的少量のアコニット酸が検出され、耐性における作用は弱く、オオムギには特有のグラミンがあり、これがアブラムシに対する耐性の主要因に成っていると考えられた。コムギは少量のアコニット酸の含有であり、グラミンは検出されなかった。

数個の未同定の作用力不明の多量成分と摂食忌避作用の認められる微量のフェノール酸もあった。いくつかの成分が耐虫性成分であるが、アコニット酸も重要な要因と考えられた。