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Original Article

Action Mechanism of *N*-Cyanomethyl-2-chloroisonicotinamide in Controlling Rice Blast Disease

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Effects of *N*-cyanomethyl-2-chloroisonicotinamide (NCI) on respiration, lipid metabolism, polyamine biosynthetic enzymes, and activities of lipoxigenase (LOX) and peroxidase (POX) in rice plants were investigated in terms of resistance against rice blast disease. The respiration in glycolytic process which supplies NADPH was stimulated by submerged application of NCI in compatible plants inoculated with *P. oryzae*, whereas the respiration related to TCA cycle which is represented by oxidation of acetate was not affected. Incorporation of [2-^{14}C]acetate into lipids was enhanced remarkably in the NCI-treated, inoculated plants at 36 hr after infection. Activities of ornithine decarboxylase (ODC) and S-adenosyl-methionine decarboxylase (SAMDC) were suppressed about 50% in inoculated plants, whereas such suppression was not observed in NCI treated plants. No remarkable change in ODC and SAMDC activities may account for steady resistance of the plants against the invading pathogens. The activities of LOX and POX were significantly augmented by infection of NCI-treated plants, which appeared to be shifted to an incompatible variety. These results indicate that NCI has a priming effect related to the resistant reaction of rice plants to the pathogen.

INTRODUCTION

N - Cyanomethyl - 2 - chloroisonicotinamide (NCI) is a nonfungicidal anti-rice blast agent.[1] Irrespective of the non-antifungal activity against *P. oryzae in vitro,* NCI was good in controlling rice blast disease when applied to rice plants *via* roots.[1] In rice plants treated with NCI, extension of invading mycelia was inhibited, and typical minute brownish lesions were formed.[1] The lesion-type rice blast appeared to be closely involved in the resistance of rice plants against infection with *P. oryzae*.[2] Such action of NCI on the host plants implied induction or enhancement of defence reaction of the plants against the disease. In general, plants respond to pathogen infection by producing physical and chemical barriers.[3] Although even susceptible plants possess such a mechanism necessary for resistance, it is considered to be not activated to a sufficient magnitude and speed to restrict the infection.[4]

We have studied the action mechanism of NCI in terms of host-parasite reaction. This paper deals with effects of NCI on some biochemical responses associated with disease resistance of rice plants, *i.e.*, respiration, lipid metabolism, polyamine biosynthesis, and activities of lipoxigenase (LOX) and peroxidase (POX).

MATERIALS AND METHODS

1. *Rice Plant and Fungus*

Two rice cultivars, Musashikogane and Aichiasahi, were used in this experiment. Rice seedlings were raised with soil mixture in plastic pots. The isolates of *Pyricularia oryzae* CAV. used were P 2 (race 003) and Hoku

1 (race 007). Both strains were compatible with Musashikogane and Aichiasahi.

2. Treatment with NCI

Method 1: Potted rice seedlings (Musashikogane) in the 3.5th leaf stage were submergedly treated with granulated NCI at a rate of 240 g a.i./10 a and cultivated in a greenhouse for 10 days.

Method 2: Seedlings in the 2nd leaf stage in a pot (90 ml in volume) were placed in a plastic cup (150 ml in volume) containing 50 ml of NCI (2.5 mg) suspension and cultivated for 5 days.

3. Inoculation

The pathogen was inoculated by spraying spore suspension of *P. oryzae* to the plants. The plants were incubated at 25 °C for 17 hr in a moist chamber and transferred to the greenhouse.

4. Respiration

Rice plants prepared by Method 1 were washed with water to remove soil particles and inoculated with P 2 (race 003, compatible). Twelve hours after inoculation, three rice plants were transferred to culture solution (30 ml) containing 37 kBq of [1-^{14}C]acetate (0.1 mM) or *d*-[U-^{14}C]glucose (0.1 mM) in a glass jar and kept in the dark. The glass jar was continuously ventilated with air, and $^{14}CO_2$ was trapped in 0.5 N NaOH solution (100 ml). Radioactivity in aliquots of the trapping solution was determined by liquid scintillation counting (LSC).

5. Lipid Metabolism

Inoculated rice plants prepared by Method 1 were cultivated in culture solution (30 ml) containing 37 kBq of [1-^{14}C]acetate (0.1 mM). The leaves were harvested at designated intervals, and incorporation of [1-^{14}C]acetate into lipids was measured. The lipids were extracted according to the method of Bligh.[5]

6. Measurement of Enzyme Activities

Inoculated leaves prepared by Method 1 or Method 2 were harvested at indicated times, and enzyme activity in the leaves was determined.

Lipoxygenase (LOX) activity was assayed as the initial rate of peroxidation of a fatty acid substrate by a procedure developed by Namai *et al.*:[6] Leaves were homogenized with a 10 times volume (w/v) of Macllvaine buffer (pH 6.4), and the homogenate was centrifuged at $3000 \times g$ for 20 min. Then 2 ml of the supernatant was added to 2 mg of sodium linolenate in 2 ml of 0.1 M Tris-HCl buffer (pH 7.5), and the mixture was incubated at 22 °C for 1 hr. After incubation, 1 ml of 0.1 N HCl was added to stop the reaction, and the mixture was extracted with 2 ml of diethyl ether. One milliliter of the ether extract was diluted with 9 ml of ethanol, and ultraviolet absorption of the solution was monitored with a spectrophotometer at 234 nm.

Activities of arginine decarboxylase (ADC), ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) were determined by measuring the $^{14}CO_2$ released from indicated substrates according to a method modified from Akiva Apelbaum's:[7] Leaves were homogenized with a 4 times volume (w/v) of 0.25 M Tris-HCl (pH 8.0) containing 25 μM pyridoxal phosphate and 10 mM DTT. The homogenate was centrifuged at $10{,}000 \times g$ for 20 min at 4 °C. One hundred microliters of the supernatant was added to a reaction mixture containing one of the indicated substrates, 8 n moles pyridoxal phosphate and 0.32 μmoles DTT in 0.2 ml of 125 mM Tris-HCl (pH 8.0). The labelled substrates used were 3.7 kBq *l*-[1-^{14}C]-arginine monohydrochloride (12.7 GBq/mM; Amersham Chemical Co., U.K.) and *l*-[1-^{14}C]-ornithine hydrochloride (2.18 GBq/mM; Amersham Chemical Co., U.K.) for assay of ADC and ODC activity, respectively. They were diluted with unlabelled arginine or ornithin to give a final concentration of 1 mM. For assay of SAMDC activity, 3.7 kBq *l*-[1-^{14}C]-S-adenosylmethionine (2.04 GBq/mM; Amersham Chemical Co., U.K.) was used as a substrate without dilution with any unlabelled substrate. Each reaction was carried out in test tubes sealed with plastic caps fitted with a filter paper disc impregnated with 0.5 ml of 10% NaOH aq. The tubes were incubated at 45 °C for 30 min. The paper discs were then transferred to scintillation vials containing 20 ml of scintillation liquid (Aquazol-2, New

England Nuclear Co., U.S.A.), and their radioactivity was determined with an Aloka LSC-1000 scintillation counter.

Peroxidase (POX) activity was assayed by the increase of absorption at 420 nm using guaiacol as a substrate.[8] Leaves were homogenized with a 40 times volume (w/v) of 0.1 M acetate buffer (pH 5.0), and the homogenate was centrifuged at 13,000×*g* for 20 min. The supernatant (enzyme extract: 0.2 ml) was mixed with 3 ml of 0.1 M acetate buffer (pH 5.0) containing 0.03% of guaiacol. Enzyme reaction was started by adding 0.1 ml of 0.08% H_2O_2, and absorbance at 420 nm was recorded for 2 min at 30 °C.

RESULTS AND DISCUSSION

1. *Respiration of Rice Plants Infected with Rice Blast Fungus*

The $^{14}CO_2$ evolution rate from [1-^{14}C]acetate or *d*-[U-^{14}C]glucose in rice cultivar Musashikogane in the 3.5th leaf stage inoculated with *Pyricularia oryzae* P-2 (race 003, compatible) is respectively shown in Fig. 1 or 2. (B) in these figures shows the change of the $^{14}CO_2$ evolution rate in plants treated with *N*-cyanomethyl-2-chloroisonicotinamide (NCI) by granular application 10 days before inoculation and (C) that in those treated with probenazole.

Evolution of $^{14}CO_2$ derived from [1-^{14}C]-acetate reached a maximum at 20 hr after inoculation, and thereafter decreased gradually (Fig. 1). The $^{14}CO_2$ evolution rate obviously differed between inoculated and non-inoculated plants 20 hr after inoculation: the rates from the former were about 2.1 and 1.2 fold higher than those of the latter at 16 and 20 hr after inoculation, respectively. There was no significant difference between the NCI- or probenazole-treated plants and the control in the augmentation of respiration after inoculation.

Evolution of $^{14}CO_2$ derived from *d*-[U-^{14}C]-glucose in plants treated with NCI or probenazole increased temporarily, reaching a maximum at 24 hr after inoculation (Fig. 2). Then it quickly decreased to under the level of non-inoculated controls at 28 hr after inoculation. In untreated plants, the time-course changes in the rates were similar regardless of inoculation. This transient augmentation of respiration rate of NCI- or probenazole-treated

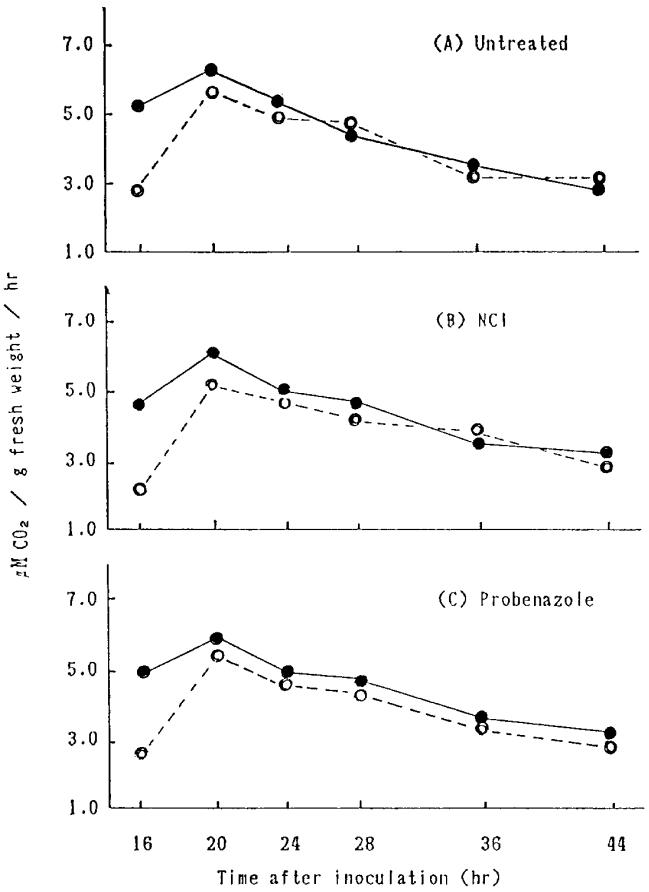


Fig. 1 Time-course of $^{14}CO_2$ evolution from [1-^{14}C]acetate in rice plants.

Rice plants were inoculated by spraying a conidial suspension of blast fungus. After incubation in a moist chamber for 12 hr, the plants were transferred to cultured solution containing 0.1 mM ^{14}C-acetate and kept in the dark.
Cultivar: Musashikogane. Inocula: P 2 (race 003).
●, Inoculated; ○, Non-inoculated.

plants at 24 hr after inoculation was observed in three separate experiments. Sekizawa[9] observed that the time of maximum rate and the half-life time of respiration burst shifted as in an incompatible combination even in a compatible infection by the application of probenazole. In incompatible combination, rice plants showed a rapid but transient augmentation of respiration by being infected with rice blast fungus.[10] Although the increased rate of respiration at 24 hr was not so significant in the above experiment, these results indicate that the metabolic turnover of glycolytic pathway in the infected plants was affected by the application of NCI or pro-

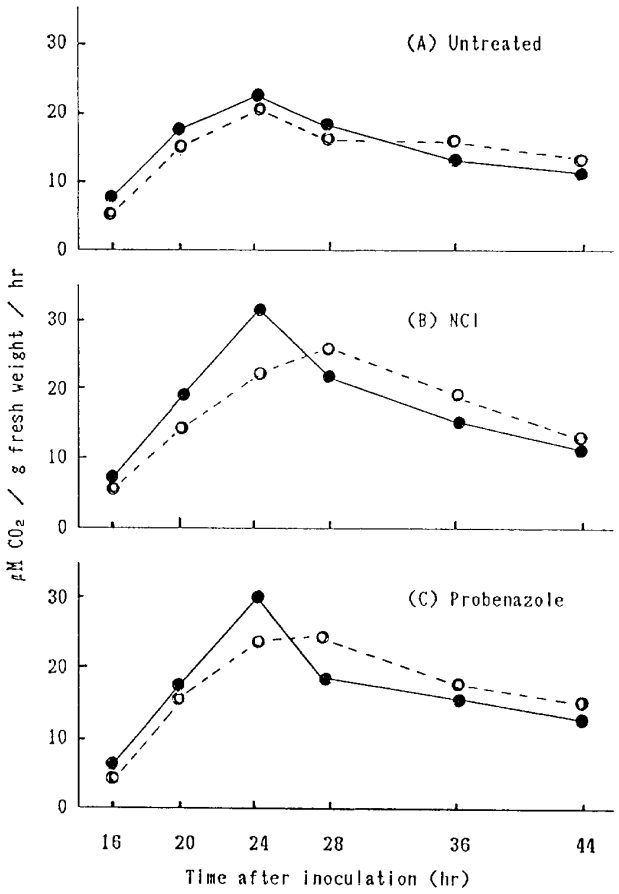


Fig. 2 Time-course of $^{14}CO_2$ evolution from *d*-[U-^{14}C]glucose in rice plants.

Cultured solution contained 0.1 mM ^{14}C-glucose. Other conditions were the same as in Fig. 1.
Cultivar: Musashikogane. Inocula: P 2 (race 003).
●, Inoculated; ○, Non-inoculated.

benazole. Plants appeared to react to inoculation with an increased biosynthetic activity and eventually cause changes in the rate of respiration.

In many cases an increase in the respiration rate has been observed at an earlier stage after inoculation in resistant than in susceptible plants.[11,12] It suggests that disease resistance based on incompatibility between host plant and pathogen may be associated with energy-requiring processes. It should be noted that our present data indicates that the rice plant respiration in glycolytic process which can supply NADPH was stimulated by fungal infection in compatible plants treated with NCI or probenazole, while respiration related to TCA cycle, which is represented by oxidation of acetic acid, was not affected.

2. *Effect of NCI on Lipid Metabolism in Rice Plants Infected with Rice Blast Fungus*

Quantitative changes in [1-^{14}C]acetate incorporation into the total lipids in rice plants are shown in Table 1. In response to infection incorporation of [1-^{14}C]acetate into the total lipids decreased to about 57% of the control after 12 hr of inoculation in each experimental section. This decrease could be, at least in part, due to the enhanced utilization of [1-^{14}C]-acetate for respiration. At 36 hr after inoculation, incorporation in untreated plants

Table 1 Incorporation of [1-^{14}C]acetate into lipids in rice leaves.

Time after inoculation (hr)	Incorporation	μg ^{14}C-acetate Eq/g fresh weight					
		Untreated		NCI		Probenazole	
		Non-IRL	IRL	Non-IRL	IRL	Non-IRL	IRL
12	Lipids	1.11	0.57	0.90	0.62	1.11	0.57
	Others	5.46	5.64	6.85	5.62	5.94	5.90
	Total	6.57	6.21	7.75	6.24	7.05	6.47
36	Lipids	1.08	1.10	1.09	1.62	0.86	1.64
	Others	6.26	6.22	6.32	7.52	5.90	6.78
	Total	7.34	7.32	7.41	9.14	6.76	8.42

Conditions of inoculation and incubation were the same as in Fig. 3. Plants were transferred to cultured solution containing 0.1 mM ^{14}C-acetate after 12 or 36 hr of inoculation and kept in the greenhouse for 7 hr.
Cultivar: Musashikogane. Inocula: P 2 (race 003). IRL: Inoculated rice leaves.
Others denote water-soluble substances.

recovered to the level in uninfected plants. In contrast, a marked increase was observed in NCI- or probenazole-treated plants (150% for NCI and about 190% for probenazole). It has been reported that total lipid or free-sterol concentrations increased in diseased or damaged tissues of higher plants, although the lipid or sterol content in different varieties of plants was not related to their susceptibility or resistance to the pathogens.[13,14] In sweet potatoes, incorporation of [1-^{14}C]acetate into lipids was enhanced several fold during the first 4 hr after cutting.[15] Kuć described that the rapid synthesis of fatty acid and their incorporation into new membranes may be a key factor of the repair mechanism to return the tissue normal.[16] Accelerated fatty acid metabolism was observed by Ohata in a resistant cultivar of rice plants infected with *Cochliobolus miyabeanus*.[17] The increase of ^{14}C-lipid accumulation in NCI-treated rice plants at 36 hr after inoculation may be correlated to resistant reaction of the plants against blast infection.

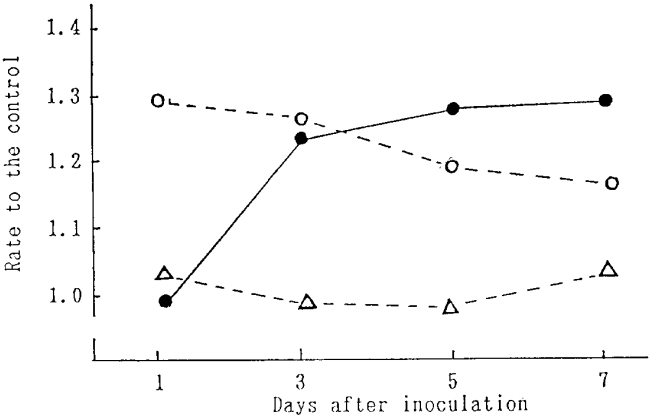


Fig. 3 Time-course determination of lipoxygenase activity in blast-infected rice leaves.

Rice plants were inoculated by spraying a conidial suspension of blast fungus. After incubation in a moist chamber for 17 hr, the plants were transferred to a greenhouse. LOX activity was indicated as the ratio to the standard activity in NCI-untreated leaves with no inoculation.
Cultivar: Musashikogane. Inocula: P 2 (race 003).
○, NCI treated; ●, NCI untreated; △, NCI treated, non-inoculated.

3. *Enzymes Activity*

Time-course determination of lipoxigenase (LOX) activity in rice leaves (3.5th leaf stage, Musashikogane) treated with NCI or inoculated with strain P-2 is shown in Fig. 3. In the leaves from plants treated with NCI, LOX activity was remarkable within 24 hr after inoculation and gradually declined, while the activity continued to increase until 7 days in the inoculated, untreated leaves.

LOX, which is active in wounded tissues,[6,18] may be one of the primary defence factors functioning in most plants, and its products can act as direct antimicrobial agents[19] or elicitors of disease resistance mechanisms.[20] The activity of LOX in rice leaves was enhanced by infection of blast fungus and accelerated by treatment with NCI.

Figure 4 indicates changes in arginine decarboxylase (ADC), ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) activities in NCI-treated and untreated leaves (3.5th leaf stage, Musashikogane) after inoculation. ADC activity did not change significantly during infection to leaves. Changes in ODC activity in both NCI-treated

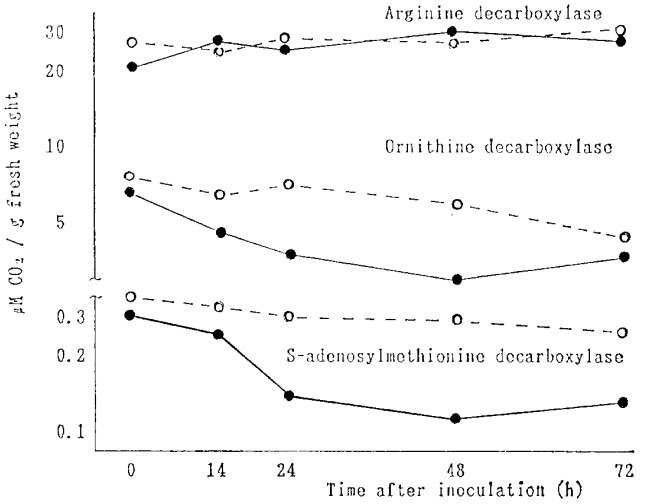


Fig. 4 Changes in the specific activity of various enzymes involved in polyamine synthesis in blast-infected rice leaves.

Conditions of inoculation and incubation were the same as in Fig. 3. Enzyme activity was shown by the amount of $^{14}CO_2$ production.
Cultivar: Musashikogane. Inocula: P 2 (race 003).
○, NCI treated; ●, NCI untreated.

and untreated leaves were very similar to those in SAMDC activity. ODC activity in untreated leaves began to decrease at 14 hr after inoculation, reaching the lowest level (about 50% of that in non-treated ones) at 48 hr. In contrast, the activity in NCI-treated leaves only slightly decreased during 72 hr. The significance of this with regard to resistance or susceptibility to infection is unknown, but it has shown that polyamine metabolism is extremely sensitive to changes in the external environment.[21] The putrescine content of oat-leaf cells and protoplasts increases within 6 hr of exposure to osmotic stress.[22] Increased ADC activity parallels the rise in putrescine, whereas ODC activity remains unchanged.[22] The decrease of ODC and SAMDC activity in blast-infected leaves without treatment is possibly the reflection of physiological disorder or inhibition of metabolism in host cells caused by infection of pathogen. No remarkable change of such activity caused by NCI may account for steady resistance of the plants against the invading pathogens in our experiment.

Figure 5 shows changes in peroxidase (POX) activity after inoculation with Hoku 1 in the top leaves of plants treated or non-treated with NCI. In the NCI-treated plant leaves, POX activity increased rapidly after inoculation, reached an maximum within 24 hr, and declined thereafter. In the NCI-untreated leaves, increase of the activity delayed but continued up to 7 days. It has been reported that POX activity rapidly increased in rice leaves within 24 hr after inoculation in an incompatible combination of rice cultivar and blast fungus race, while it increased slightly or did not increase in a compatible combination at the early stage of infection, but gradual increased 3–5 days after inoculation, eventually exceeding that of an incompatible combination.[23] By NCI treatment, the pattern of enzyme activity in Aichiasahi leaves inoculated with a compatible race appeared to be converted to that in cultivar leaves in incompatible combination. The enhancement of POX activity is reported to be closely related to the resistant reaction of the host plant to a pathogen,[24] with POX acting as a catalyst of lignin biosynthesis. Kuć and co-workers have showed that resistant cucumbers were rapidly lignified when infected by *C. cucumerinum*, particularly in cells adjacent to the site of invasion. Lignification was also observed in susceptible cucumbers, but the reaction was delayed until after the pathogen had ramified into the tissue.[25,26] Thus timing of increase in POX activity appeared critical to resist the development of the pathogen in the host plants.

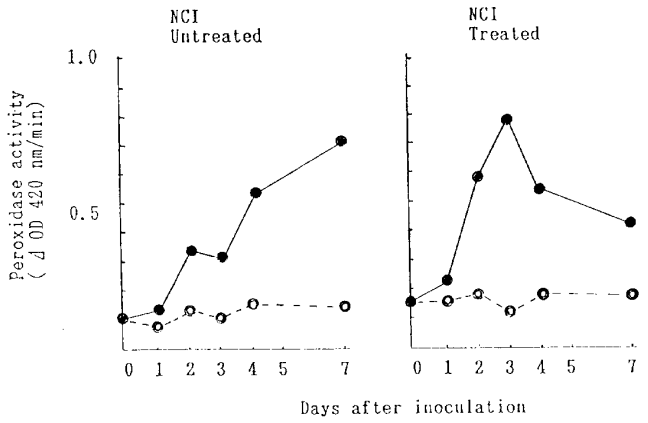
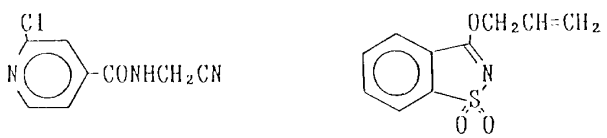


Fig. 5 Time-course determination of peroxidase activity in blast-infected rice leaves.

Conditions of inoculation and incubation were the same as in Fig. 3. POX activity was shown by the increase of absorption at 420 nm per minute. Cultivar: Aichiasahi. Inocula: Hoku 1 (race 007). ●, Inoculated; ○, Non-inoculated.

When rice plants were treated with NCI by submerged application and subsequently inoculated with *P. oryzae*, the respiration and lipid metabolism in the host plants were enhanced and the activities of LOX and POX were accelerated or increased. These facts suggest that the mode of action of NCI in rice plants is similar to that of probenazole, which is known as an anti-rice blast agent that induces the resistance in host plants.[27,28] However, NCI and probenazole have no similarity in their chemical structures (Fig. 6). Further study to elucidate the action mechanism is in progress.



NCI Probenazole

Fig. 6 Chemical structures of NCI and probenazole.

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要　　約

イネいもち病防除における *N*–シアノメチル–2–クロロイソニコチンアミドの作用機構

瀬口宏一郎，黒滝美音子，関戸茂子，山口　勇

N-Cyanomethyl-2-chloroisonicotinamide (NCI) は非殺菌性のイネいもち病防除化合物である．プラスチックポットに育成した 3.5 葉期のイネに NCI を水面施用し，イネいもち病菌分生胞子を接種すると 16 時間後からイネ葉の呼吸が上昇した．^{14}C-酢酸を基質とした場合，NCI 処理区と無処理区で $^{14}CO_2$ の生成増に顕著な差はなかった．一方，^{14}C-グルコースを基質とすると，感染初期に NCI 処理区で一時的な $^{14}CO_2$ の生成増が認められた．また，粗脂質への ^{14}C の取り込みは，感染 36 時間後には NCI 処理区で増加が認められた．ポリアミン生合成に関与するアルギニン脱炭酸酵素活性は NCI 処理に関係なく胞子接種後いずれも経時的に微増したが，オルニチン脱炭酸酵素活性と S–アデノシルメチオニン脱炭酸酵素活性は NCI 処理区ではほとんど変動せず，無処理区では減少した．これらの結果は NCI によって罹病時のイネ体における解糖系と脂質代謝が活性化されていることを示唆している．また，ポリアミン代謝はストレスに対して敏感に反応することから，NCI 処理イネ体における感染後のポリアミン生合成関連酵素活性減少の阻害は，イネ体の感染による組織ダメージが抑制されていることを示している．リポキシゲナーゼ活性とペルオキシダーゼ活性は NCI 処理によってその合成時期が速められ，非親和性の組合せで見られるような消長を示した．