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Original Article

Effect of Pencycuron on the Osmotic Stability of Protoplasts of *Rhizoctonia solani**

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Pencycuron shows a specific inhibitory activity on the mycelial growth of *Rhizoctonia solani*. Even in the same anastomosis groups (AGs), *e.g.* AG4, there are both isolates sensitive and less sensitive (inherently resistant) to pencycuron. The regeneration of colonies from protoplasts of R-C (pencycuron-sensitive isolate in AG4) significantly decreased by the osmotic shock in the presence of pencycuron, while such effect was cancelled by washing off the chemical prior to the osmotic shock. However, in Rh-131 (a less sensitive isolate in AG4), the application of the chemical appeared to stimulate the regeneration from protoplasts. Further the measurement of optical density of protoplasts suspensions was performed to elucidate the effect of pencycuron on the osmotic stability of protoplasts in a short period. The optical density of suspensions of R-C protoplasts rapidly declined in a short period of incubation by the osmotic shock in the presence of pencycuron, but the effect on Rh-131 protoplasts was not statistically significant regardless of the presence or the absence of the chemical. These results suggest that the cell membrane of the pencycuron-sensitive isolate (R-C) is specifically affected by pencycuron.

INTRODUCTION

Rhizoctonia solani KÜHN is a troublesome phytopathogen infecting a number of economically important crops, including rice plants and potatoes.[1] For the control of the plant diseases caused by *R. solani*, such as rice sheath blight and potato black scurf, pencycuron (1-(4-chlorobenzyl)-1-cyclopentyl-3-phenylurea) has been used worldwide since its development in 1985.[2] In laboratory experiments, pencycuron exerts its specific activity to inhibit the mycelial growth of *R. solani* at different concentrations depending on the anastomosis groups (AGs): the sensitivity to pencycuron was observed in AG1, 2, and 3 of *R. solani*, but AG5 was shown to be far less sensitive. Interestingly, it appeared that there are both pencycuron-sensitive and resistant (less sensitive) isolates in AG4. Thus, pencycuron has an extremely narrow antimicrobial spectrum even in the same anastomosis group of *R. solani*. Yamada *et al.* reported that pencycuron neither inhibited the biosyntheses of sterol and chitin nor the activity of trehalase in *R. solani*.[2] While pencycuron had no significant effects on other macromolecule biosynthesis such as nucleic acids and proteins in *R. solani*, Ueyama *et al.* found remarkable changes in the mycelial morphology and in the cytoskeletal microtubules of the hyphal tips of the sensitive isolate to be caused by pencycuron.[3] Pencycuron, however, appeared not to exhibit conspicuous inhibitory effects on a nuclear division in *R. solani* as was reported for such antimicrotubule agents as carbendazim and thiophanate-methyl.[4] Although the detailed action mechanism of pencycuron on cytoskeletal microtubules still remains obscure, findings by Ueyama *et al.* suggest that the primary target site of pencycuron may be around the plasma membrane even if the chemical has no direct effects on microtubule formation.

This paper deals with the effects of pencycuron on the osmotic stability of protoplasts from sensitive and resistant isolates of *R. solani*, focusing on the effects of the chemical on the function of the cell membranes.

* Action Mechanism of Pencycuron, a Specific Antifungal Compound (Part 1).

MATERIALS AND METHODS

1. *Culture Method*

Isolates of *R. solani* AG4 were kindly supplied by National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries. All isolates were cultured on potato dextrose agar (PDA) at 25°C for 3 days and maintained at 4°C for storage.

2. *Inhibitory Activity of Pencycuron on the Mycelial Growth of R. solani*

The sensitivity of *R. solani* to pencycuron was evaluat-

ed by the mycelial growth on PDA with or without the chemical. Pencycuron was dissolved in dimethyl sulfoxide (DMSO) and added to autoclaved PDA after cooling to 50–55°C. The concentration of DMSO was less than 1% (v/v) in all media. Mycelial plugs (diameter, 5 mm) from the 3 day-cultured actively growing colonies were centrally inoculated on the PDA and the mycelial growth was determined by measuring the diameter of colonies after 3 days incubation at 25°C.

3. *Preparation of Protoplasts of R. solani*

Protoplasts of *R. solani* were prepared according to the modified method of Hashiba & Yamada.[5] Mycelia grown for 24 hr in potato dextrose liquid medium were harvested by centrifugation at 3000×*g* for 10 min, followed by washing with 0.6 M mannitol. Then they were suspended in 10 ml of enzyme solution containing 7 mg/ml Novozyme and 60 μl/ml β-glucuronidase in 0.6 M mannitol, and incubated on a reciprocal shaker at 70 strokes per minute at 34°C for 3 hr. The protoplasts were filtered through a 20 μm nylon mesh to remove mycelial fragments, and the filtrate was centrifuged in 0.6 M mannitol three times at 1000×*g* for 5 min to wash off the enzymes.

4. *Effect of Pencycuron on the Osmotic Stability of Protoplasts*

The effect of the osmotic shock on protoplasts was examined by changing the concentration of mannitol as osmotic stabilizer from 0.6 to 0.2 M by the addtion of sterilized distilled water. As shown in Fig. 1, the protoplasts were either subjected to osmotic shock in the last 3 min of a 10 min-treatment of 1 μg/ml pencycuron, or the 3 min-osmotic shock was given after the 10 min treatment of pencycuron, followed by successive washing of the chemical by centrifugation at 1000×*g* for 5 min in 0.6 M mannitol. Protoplasts in the regeneration medium (PDA containing 0.6 M mannitol) were incubated at 25 °C for 3 days, and the numbers of colonies appeared on each plate were counted. The survival percentage of protoplasts was calculated as follows.

Survival percentage=
(Numbers of colonies regenerated from protoplasts treated with pencycuron and osmotic shock/Colony numbers from non-treated protoplasts)×100

For determination of the effects of pencycuron on the osmotic stability of protoplasts in a short period, the reduction of optical density of the protoplast suspension was directly measured at 600 nm with a spectrophotometer (Shimadzu UV-265) after the osmotic shock was given to protoplasts.

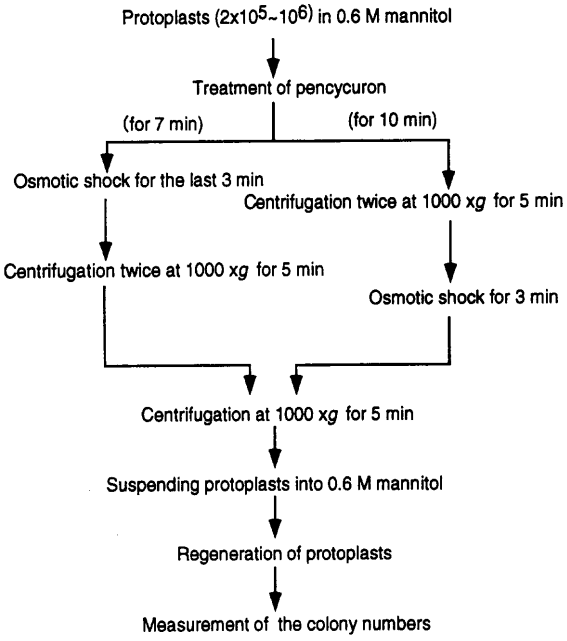

Fig. 1 Procedures for measuring the effect of pencycuron on osmotic stability of protoplasts of *Rhizoctonia solani*.

RESULTS

1. *The Effect of Pencycuron on the Mycelial Growth of R. solani*

The inhibitory activity of pencycuron on the mycelial growth of isolates of *R. solani* which belong to the same anastomosis group (AG4) was examined. As shown in Table 1, they were distinctly divided into pencycuron-sensitive isolates (R-C, BO-3, and GM-11) and less sensitive (inherently resistant) ones (Rh-131, SH-34, and K-63). The result suggests that there is no direct relationship between the pencycuron sensitivity and anastomosis characters in *R. solani*, although the sensitivity was roughly correlated to anastomosis behavior.[2] Thus, in this study, R-C (sensitive isolate) and Rh-131 (less sensitive isolate) were selected as test organisms to elucidate the action mechanism of pencycuron.

2. *Survival Ratio of Protoplasts by the Osmotic Shock*

Osmotic stabilities of R-C and Rh-131 were tested by

Table 1 The inhibitory effects by pencycuron on mycelial growth of *Rhizoctonia solani* AG4s.

Isolates	Concentration of pencycuron (μg/ml)			
	0.008	0.04	0.2	1.0
R-C	19.0[a]	60.7	81.6	100.0
BO-3	6.6	25.0	61.6	100.0
GM-11	24.5	60.1	82.5	100.0
Rh-131	8.0	11.6	15.7	22.1
SH-34	1.1	0.9	5.1	20.5
K-63	6.0	7.0	17.9	39.3

[a] Numbers are inhibitory percents calculated from the colony diameters cultivated on PDA with or without pencycuron for 3 days after inoculation. Values are means from three experiments.

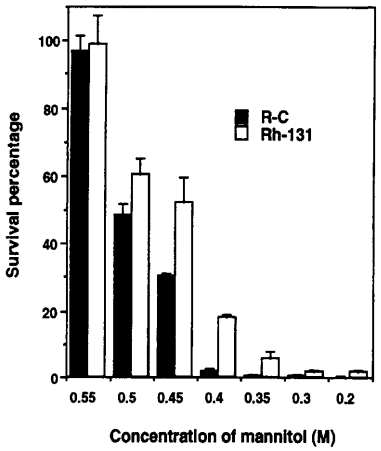

Fig. 2 The osmotic stability of protoplasts of *Rhizoctonia solani* R-C and Rh-131.

The protoplasts were subjected to osmotic shock for 3 min and incubated on PDA containing 0.6 M mannitol for 3 days. The stability was indicated by the comparison of numbers of colonies regenerated from treated protoplasts over those from protoplasts kept at 0.6 M mannitol (control).

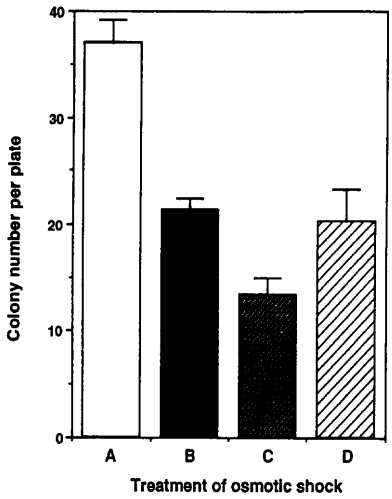

Fig. 3 The effect of pencycuron on the osmotic stability of protoplasts of *Rhizoctonia solani* R-C.

Osmotic shock was given by adjusting mannitol concentration to 0.5 M. The protoplasts were subjected to osmotic shock for 3 min in the absence or presence of 1.0 μg/ml pencycuron. A: control (no treatment of pencycuron and osmotic shock), B: only osmotic shock was given, C: osmotic shock was given in the last 3 min of 10 min-treatment of pencycuron, D: osmotic shock was given after washing off pencycuron.

the application of osmotic shock to their protoplasts (Fig. 2). Sterilized distilled water was added to the original suspensions of protoplasts in 0.6 M mannitol and the suspensions were gently shaken. At 0.5–0.35 M of mannitol, the differences of the survival percentage of protoplasts between R-C and Rh-131 were statistically significant. Rh-131 (pencycuron-less sensitive) protoplasts showed higher stability to osmotic shock than the protoplasts from R-C (sensitive to pencycuron). As compared with the numbers of colonies regenerated from the protoplast kept in 0.6 M mannitol, a notable decrease of survival ratio was firstly observed at 0.5 M of mannitol in both R-C and Rh-131. Since the difference of survival ratio between R-C and Rh-131 was comparatively small at 0.5 M mannitol, the effects of pencycuron on osmotic stability of protoplasts were hereafter mostly examined at this concentration of mannitol.

3. *Effect of Pencycuron on the Osmotic Stability of Protoplasts*

Following Fig. 1, osmotic shock by 0.5 M mannitol was applied to determine the osmotic stability of protoplasts in the presence or absence of 1.0 μg/ml pencycuron. Figure 3 shows that the survival percentage of protoplasts from *R. solani* R-C was 57.6% when only the osmotic shock was given to the protoplasts for 3 min (B). With the application of osmotic shock in the last 3 min of a concurrent 10 min pencycuron treatment, the survival percentage decreased to 36.1% (C). However, no significant decrease of survival percentage was observed if the chemical was washed off before the osmotic shock (D).

The number of colonies regenerated from R-C protoplasts which were osmotically shocked in 0.5 M mannitol in the presence of pencycuron decreased in a dose-response manner as shown in Fig. 4. However, the colony numbers from Rh-131 increased quite remarkably by the treatment with pencycuron. Since the actual ratios of colony formation from protoplasts were low in *R. solani* even under the standard conditions where

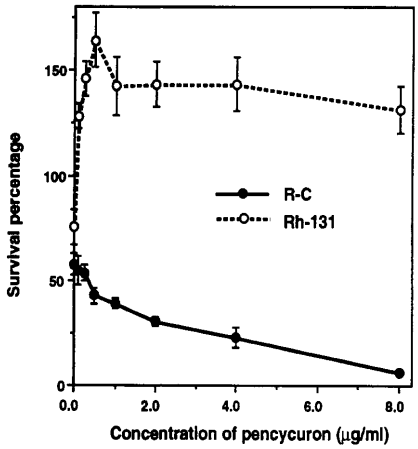

Fig. 4 The effect of pencycuron on the colony formation from protoplasts of *Rhizoctonia solani* R-C and Rh-131.

The osmotic shock was given by reducing the mannitol concentration to 0.5 M for the last 3 min in 10 min treatments of various concentrations of pencycuron. The number of colonies regenerated on PDA containing 0.6 M of mannitol were counted after 3 days incubation at 25°C and compared with the colony numbers in control.

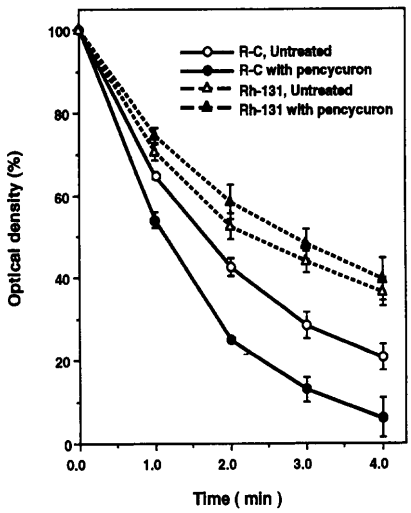

Fig. 5 The effect of pencycuron on the osmotic stability of protoplasts from *Rhizoctonia solani* R-C and Rh-131.
The osmotic stability was determined by measuring the optical density of protoplast suspensions with a spectrophotometer at 600 nm. In the presence of 1.0 μg/ml pencycuron, the protoplasts were subjected to osmotic shock by adding distilled water to adjust osmosis to 0.45 M mannitol.

protoplasts were kept at 0.6 M mannitol and inoculated onto 0.6 M mannitol-PDA without any treatment (2.5% in R-C and 6.1% in Rh-131, respectively), the results may imply that pencycuron specifically stimulated the colony formation from the survived protoplasts from Rh-131 in 0.5 M mannitol.

The effect of pencycuron on the osmotic stability of protoplasts during a short period was examined with the spectrophotometer by directly measuring the optical density of the protoplast suspensions at 0.45 M mannitol. While the optical density declined sharply in the protoplast suspension of R-C by the treatment with pencycuron, its effect on the protoplasts from Rh-131 was not significant (Fig. 5). Such fluctuation of optical density was not observed in the isotonic 0.6 M mannitol suspensions of both isolates regardless of the presence or absence of pencycuron. Among the osmotic shocks given by 0.4, 0.45, and 0.5 M mannitol, the effect of pencycuron on the osmotic stability of R-C protoplasts was the highest in 0.45 M (data not shown). On the contrary, effects of pencycuron on the protoplast suspensions of Rh-131 were not statistically significant at all the osmotic shock applications tested. However, this does not imply that protoplasts of Rh-131 are essentially different from those of R-C because both protoplasts from R-C and Rh-131 isolates showed an abrupt decrease of optical density within 1 min if they were suspended in distilled water. It appears that the plasma membrane from R-C isolate has some specific target sites sensitive to pencycuron.

DISCUSSION

Pencycuron showed a different inhibitory activity on the mycelial growth of *R. solani* isolates which belong to AG4. Isolate R-C was quite sensitive to the chemical at concentrations below 1.0 μg/ml but Rh-131 was much less sensitive. It suggests that the action mechanism of pencycuron is tremendously specific and the anastomosis in *R. solani* does not directly correlate with pencycuron sensitivities. Since we could not obtain resistant mutants from R-C isolate in laboratory, the less sensitive isolate Rh-131 in AG4 was used for the experiment in this study.

Leroux & Gredt reported that some carbendazim-resistant strains of *Botrytis cinerea* and *Pseudocercosporella herpotrichoides* exhibited a higher sensitivity to pencycuron.[6] While their results were not confirmed in our experiments, Ueyama *et al.* observed similar morphological changes to occur by the application of pencycuron and carbendazim to R-C isolate of *R. solani*.[3] However, they found a remarkable difference between the effects of these compounds; carbendazim conspicuously inhibited the nuclear division of *R. solani*, whereas pencycuron caused only deterioration of microtubule arrays to work as cytoskeleton in its hyphal tips.

Young also reported that zarilamide, a fungitoxic compound with a broad spectrum on Oomycete fungi, caused an abnormal nuclear division and the change of microtubule cytoskeleton both in *Phytophthora capsici* and in tobacco suspension-cultured cells, suggesting that it acts as an antimicrotubule agent.[7] The mode of action of pencycuron seemed to have some similarity with that of zarilamide in that both chemicals bring about a rapid loss of normal microtubule filament in the hyphal tips of sensitive fungi. However, it is plausible that pencycuron has a different target site from zarilamide because pencycuron exerts no significant inhibitory activity on nuclear division in *R. solani*. Cytoskeleton fibers comprised microtubules, actin filaments and intermediate connecting proteins play an important role to maintain the eukaryotic cell structure.[8] If these cytoskeleton fibers are disrupted, directly or indirectly, the function and stability of cell membranes must be greatly affected, and *vice versa*.

In the present study, osmotic stability of protoplasts proved to be significantly affected by pencycuron in the sensitive isolate R-C of *R. solani*. However, the regeneration of colonies from the protoplasts recovered remarkably when osmotic shock was given after washing off the chemical, suggesting that enough amounts of pencycuron in the membrane were indispensable to affect the osmotic stability of R-C protoplasts. Heath *et al.* presented a new model for hyphal tip growth in filamentous fungi, proposing that the plasticity of the hyphal tips is regulated by the cytoskeletal microtubules containing F-actin

and spectrin which are attached to the cell membrane by integrin.[9-11] Microtubules and actin filaments are formed by polymerization of protein subunits called tubulin and G-actin, respectively, and the polymerization of such cytoskeletal fibers is dramatically controlled by the cell phase.[8] Although it is not shown that pencycuron directly inhibits the assembly of cytoskeleton fibers in sensitive *R. solani*, it is likely that pencycuron may localize in plasma membranes due to its high lipophilicity and/or bind to responsible protein(s) specifically in a sensitive isolate (R-C). Such mechanism may explain why protoplasts from R-C are osmotically fragile and the regeneration of colonies from protoplasts of Rh-131 (less sensitive isolate) is stimulated in the presence of pencycuron. Studies are now in progress on lipid composition in membranes of R-C and Rh-131 as well as the effects of pencycuron on microtubule formation *in vitro*.

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要　　約

Rhizoctonia solani のプロトプラストの浸透圧耐性に及ぼすペンシクロンの影響

金　興泰，鎌倉高志，山口　勇

ペンシクロンはきわめて作用特異性の高い薬剤で，*Rhizoctonia solani* の同一菌糸融合群（AG4）に対してもその作用に顕著な差異が認められる．菌糸融合群4に属するR-C株（ペンシクロン感受性）およびRh-131株（同薬剤非感受性）を選び，病原菌の細胞膜強度に及ぼすペンシクロンの影響を検討したところ，R-Cではプロトプラストからのコロニー再生率が薬剤存在下での低浸透圧処理により対照区（低浸透圧処理のみ）に比べて有意に減少した．しかし，薬剤を洗浄後，低浸透圧処理すると対照区と同等なコロニーの再生率を示した．一方，Rh-131ではプロトプラストからの再生率は低浸透圧処理により減少するが，ペンシクロン存在下におけるコロニーの形成は逆に促進されるような結果が得られた．ペンシクロン処理直後の短時間の間の薬剤の細胞膜に及ぼす影響をプロトプラスト懸濁液の吸光度の変化を測定して調べたところ，R-Cのプロトプラスト懸濁液に薬剤存在下で低浸透圧処理すると懸濁液の吸光度は顕著に減少したが，Rh-131のプロトプラスト懸濁液での低浸透圧処理の効果はペンシクロンの存在によって有意な影響を受けなかった．これらの結果からペンシクロンは感受性菌の細胞膜に特異的に影響を与えることが示唆された．