Rhizoctonia solani Kuhn (AG-1, IA)の菌核形成に及ぼすリンおよびマグネシウムの影響

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The Effects of Phosphorus and Magnesium on Sclerotium Formation in *Rhizoctonia solani* Kühn

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Abstract

None or very few sclerotia of *Rhizoctonia solani* (AG-1, IA) were formed on Hopkins agar medium without KH_2PO_4, though hyphae developed in some extent. While, the number and weight of sclerotia were decreased when $MgSO_4$ was absent. As KH_2PO_4 in the basal medium was replaced with other phosphates different in cation, sclerotia were well formed. The optimal concentration of phosphate as KH_2PO_4 for the sclerotium formation, in number and weight, and hyphal development was in the region of 100 ppm. The addition of magnesium resulted in the increase of the number and weight of sclerotia proportionally with the amount of the magnesium compound. The results indicate that phosphorus ion is indispensable to the sclerotium formation, whereas magnesium ion is promotive. The absence of phosphorus at the maturing stage resulted in the formation of few sclerotia, even though phosphorus was presented at the hyphal and initial phases. While defficiency of phosphorus in the early stage of sclerotium development showed no effect on the number and weight of sclerotia formed, if the phosphorus was supplied in subsequent periods. The branching internodes of hyphae grown on the phosphorus free medium were longer than those of on the medium containing phosphorus. This decrease of the hyphal branching will lead to the decrease of interweaving hyphae and will result in the scarce sclerotium formation. A large accumulation of ^{32}P to sclerotia was strikingly observed in this experiment.

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Key words: *Rhizoctonia solani*, sclerotium, inorganic ions, ^{32}P translocation, autoradiography.

INTRODUCTION

Some of the soil-borne fungi produce sclerotia as a resting structure against various adverse environmental factors. It is well known that fungal sclerotium production is affected by various physical and chemical factors[2,5,9,10,14,15,17,18,25].

In nutrients, especially, the quality and quantity of carbon and nitrogen sources and C/N ratio take an important role in the sclerotium production. It was reported that microsclerotium formation of *Verticillium albo-atrum* was promoted by manganese ion and that certain isolates of *Sclerotinia sclerotiorum* did not produce sclerotia without zinc ion[8,24]. In general, fungal morphogenesis such as spore, myxospore, perithecium and stroma formation is induced or promoted by various inorganic ions such as potassium, calcium, magnesium, manganese and phosphorus ions[26].

In this study, the phosphorus and magnesium requirements for the sclerotium formation of *Rhizoctonia solani* were examined. Furthermore, in order to elucidate the association of the phosphorus on the morphogenesis, the effect of phosphorus on the hyphal branching which

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is the first step of sclerotium formation and the manners of incorporation and transportation of phosphorus were investigated. The abstract has been reported elsewhere[19].

MATERIALS AND METHODS

Fungus. An isolate (C-324) of *Rhizoctonia solani* Kühn (NIAS) was used in this study. This isolate originally isolated from sugarcane in Kagoshima Prefecture belongs to the anastomosis group AG-1 and the culture type IA.

Media. Preculture of the fungus was conducted on potato dextrose agar (PDA). Hopkins medium (2 g KNO_3, 10 g glucose, 0.5 g $MgSO_4$, 0.1 g KH_2PO_4, 1,000 ml water) was used as basal medium. The media were adjusted to pH 6.5 by NaOH before autoclaving at 110°C for 10 min.

Exp. 1. Effects of inorganic compounds. As inorganic chemicals, one or both of $MgSO_4$ and KH_2PO_4 were removed from basal Hopkins medium or additionally amended at various concentration of the compounds (KH_2PO_4:1–1,000 ppm, $MgSO_4$: 100–5,000 ppm) to the medium. For the effect of phosphate, KH_2PO_4 of Hopkins medium was replaced with other phosphates with different cations. The edge of mycelial mat precultured on PDA was cut with a cork borer (5 mm in diam.) and centered on the various media and cultured at 25°C. The initials of sclerotia were counted at 4 days after inoculation and sclerotia formed on the media were collected, desiccated and weighed.

Exp. 2. Effect of phosphorus on each stage of sclerotium morphogenesis. The seamless cellulose tubing for dialysis was opened and cut to round shape, 8.5 cm in diameter. This membrane was sheeted on the media with or without KH_2PO_4 and then mycelial disc was centered on the membrane and precultured at 25°C. The membrane which covered with mycelia was transferred on the another media at 48 hr (Mycelial stage) and 96 hr (Initial stage) after inoculation. After the culture for 14 days at 25°C, sclerotia formed on the membrane were collected, counted, desiccated and weighed.

Exp. 3. Effect on hyphal branching. The isolate was cultured on various media amended with inorganic compounds to basal Hopkins medium. After the culture for 48 hr, the hyphal internode which is the distance from one hyphal branch to another branch was measured with a light microscope.

Exp. 4. Incorporation and transportation of phosphorus. One mCi (37 MBq) of $KH_2{}^{32}PO_4$ was diluted with distilled water to 1/200. One ml of the solution was filtered through a 0.2 μm cellulose nitrate filter and added to 15 ml of Hopkins medium, and stirred well with mixer. The medium was poured into petri dish (9 cm in diam.). After cooling, cellulose membrane (8 cm in diam.) was sheeted and the mycelial disk precultured on PDA was placed on the center and kept at 25°C in an incubator. The mycelia developed vigorously on the membrane. When the culture was reached at each stage of sclerotium development such as the initial, white sclerotium and matured sclerotium, the membrane was peeled off. Media after culture were punched out randomly with a cork borer 6.5 mm in diameter. Those samples were dried before counting to keep off the self absorption of the radioactivity. Radioactivities of samples were counted at 1,200 V for 1 min by GM counter (Aloca Co.).

Exp. 5. Autoradiography. The isolate was cultured on Hopkins medium containing ^{32}P labelled KH_2PO_4. After 14 days, X ray film (Fuji FR) was placed attaching tightly on the culture for 6 hr.

RESULTS

Effects of phosphorus and magnesium

The sclerotium initials and matured sclerotia were produced well on basal Hopkins and $MgSO_4$-absent media. While, no or very little production was observed on the media in which

two inorganic compounds or KH_2PO_4 were absent (Table 1). The optimal concentration of KH_2PO_4 for the sclerotium formation was in the region of 100 ppm at presence and absence of magnesium (Table 2). While the number and size of sclerotia increased in proportion to $MgSO_4$ concentration (Table 3).

Effects of various kind of phosphates

As KH_2PO_4 in basal medium was replaced with other phosphates such as K_2HPO_4, $(NH_4)_2HPO_4$ and $CaHPO_4$, sclerotia were well produced on each phosphorus compound medium with a certain variation on the number and total weight. In regard to the effect of potassium phosphates on the sclerotium formation, there were quite differences between KH_2PO_4 and K_2HPO_4. KH_2PO_4 showed twice as much as sclerotium forming effect than K_2HPO_4 (Table 4).

Table 1. Sclerotium formation of *Rhizoctonia solani* on various media

Media [a]	Number of initials	Sclerotia formed	
		Number [b]	Weight per dish (mg)
A	62.4±9.9	30.5±4.9	57.3±4.9
B	3.5±1.8	0.8±1.0	1.3±2.1
C	59.1±13.1	12.2±4.1	38.0±3.3
D	1.3±1.9	0.3±0.5	1.5±2.5

a) A: basal Hopkins medium, B: without KH_2PO_4 and $MgSO_4$, C: without $MgSO_4$, D: without KH_2PO_4.
b) Sclerotia formed in one dish.

Table 2. The effect of KH_2PO_4 concentration on sclerotium formation of *R. solani*

Concentration of KH_2PO_4 (ppm)	With $MgSO_4$		Without $MgSO_4$	
	Number	Weight per dish (mg)	Number	Weight per dish (mg)
0	0.3±0.5	1.5±2.5	0.8±1.0	1.3±2.1
1	0.2±0.0	2.6±1.6	0.6±1.2	1.0±1.4
10	4.9±2.8	15.8±3.6	4.8±0.8	16.2±1.8
100	30.5±4.6	57.3±4.7	12.2±4.0	38.0±3.1
1,000	13.1±2.5	28.4±4.3	10.3±3.3	34.8±3.1

Table 3. The effect of $MgSO_4$ concentration on sclerotium formation of *R. solani*

Concentration of $MgSO_4$ (ppm)	Number of initials	Sclerotia formed	
		Number	Weight per dish (mg)
0	59.1±13.1	12.2± 4.1	38.0±3.3
100	50.6± 4.1	28.6± 3.6	42.3±6.7
500	62.4± 9.4	30.5± 4.7	57.3±4.7
1,000	69.6±11.3	48.8± 9.1	54.6±4.3
5,000	106.7± 9.7	66.4±10.5	61.7±2.1

Table 4. The effect of various phosphates on sclerotium formation of *R. solani*

Phosphates	Number of initials	Sclerotia formed	
		Number	Weight per dish (mg)
KH_2PO_4	62.4±9.4	30.5±4.7	57.3±4.0
K_2HPO_4	23.6±9.1	14.4±6.1	31.0±4.0
$(NH_4)_2HPO_4$	42.7±4.2	18.1±3.7	44.7±2.0
$CaHPO_4$	42.7±4.5	20.1±1.1	37.6±2.0

Effect on hyphal growth and hyphal branching

Although there was no effect of KH_2PO_4 and $MgSO_4$ on the hyphal linear growth, the hyphal weight was increased in proportion to KH_2PO_4 concentration (Table 5). As shown in Fig. 1, when the fungus was cultured on the phosphorus free medium, the primary and secondary branching internodes were longer than that of on the phosphorus containing medium.

Table 5. The effect of KH_2PO_4 concentration on hyphal growth of *R. solani*

Concentration of KH_2PO_4 (ppm)	With $MgSO_4$		Without $MgSO_4$	
	Diameter [a]	Weight [b]	Diameter	Weight
0	8.9±0.4	7.8±1.4	7.8±0.6	10.2±2.6
10	8.7±0.3	31.8±5.1	7.5±0.8	28.5±5.4
100	8.7±0.2	63.9±4.8	8.2±0.3	34.5±1.6
1,000	8.6±0.3	55.6±2.4	8.2±0.5	32.6±3.0

a) The diameter of mycelial colony (cm) was measured at 48 hr after inoculation.
b) The hyphae (mg) were weighed at 2 weeks after inoculation.

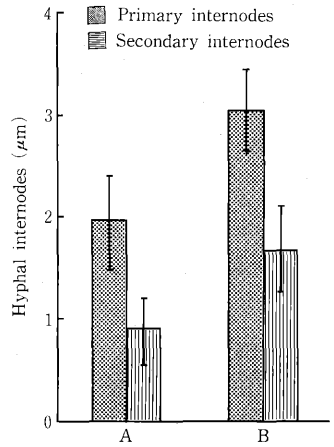

Fig. 1. The effect of phosphorus on the frequency of hyphal branching of *R. solani*. A: Hopkins medium, B: Hopkins medium without KH_2PO_4.

Table 6. The effect of KH_2PO_4 on each stage of sclerotium formation of *R. solani*

KH_2PO_4	Hyphal stage [a]		Initial stage [b]	
	Number	Weight per dish (mg)	Number	Weight per dish (mg)
Present [c] ↓ Present	14.0±1.5	38.9±5.9	11.2±1.5	42.5±5.9
Present ↓ Absent	2.3±1.0	6.4±2.1	4.4±1.2	16.0±5.5
Absent ↓ Present	12.6±1.5	37.0±6.6	20.9±2.3	42.2±5.9
Absent ↓ Absent	0.2±0.3	1.5±2.2	0.0±0.0	0.0±0.0

a) The hyphal stage: 48 hr after inoculation.
b) The initial stage: 96 hr after inoculation.
c) The media were with phosphate (Present) or without phosphate (Absent).

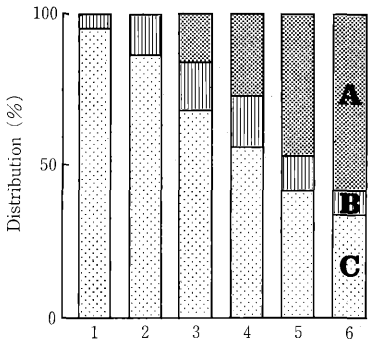

Fig. 2. Rate of ^{32}P distribution in the medium, mycelium, and sclerotium at different stage of sclerotium morphogenesis of *R. solani*. 1: Hyphal stage, 2: Initial stage, 3: White sclerotium stage, 4: Pigmenting stage, 5: Matured stage (10th day), 6: Matured stage (14th day). A: sclerotium, B: mycelium, C: medium.

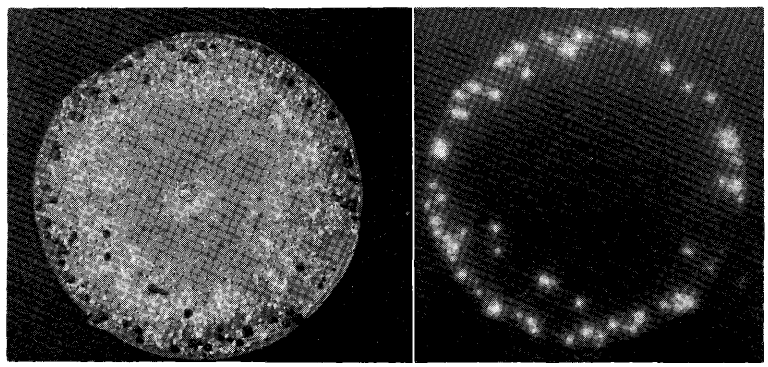

Fig 3. The sclerotium formation of *R. solani* at 14 days after culture on Hopkins medium and accumulation of ^{32}P in sclerotia formed. Left: Culture on Hopkins medium, Right: Autoradiograph.

Effects of phosphorus on several stages of sclerotium formation

No sclerotium was differentiated from hyphae on the medium without phosphorus. When phosphate was supplied at the hyphal phase (2 days after inoculation) and the hyphal mat was transferred afterwards on the phosphorus absent media, few sclerotia were differentiated (Table 6). While, even when the phosphate was absent during the hyphal stage, sclerotia were well developed when the culture was transferred on the phosphorus present medium. This phenomenon was also observed when the experiment was conducted at the sclerotium initial phase (4 days after inoculation) (Table 6). When phosphorus existed at the initial phase, *ca.* 4 sclerotia (16 mg) per a dish were differentiated even when the culture was transferred on the phosphorus absent medium. While, as the phosphorus was absent at the initial stage, *ca.* 20 sclerotia (42 mg) per a dish were formed when the cultures were transferred on the phosphorus present media.

Incorporation and translocation of phosphate

By tracing and autoradiographic investigations, almost isotope (^{32}P) detected in the medium at the hyphal phase (48 hr after inoculation) was transported to hyphae and about 60% of them was finally accumulated in the sclerotium body (Figs. 2 and 3).

DISCUSSION

The sclerotium morphogenesis of fungi was affected by nutrients and physical fac-

tors[1,2,5,6,8-10,14,16,18,20-25]. Hashiba *et al.*[6] revealed that the N/C ratio and cyclic AMP have important role for the sclerotium formation of *Rhizoctonia solani*. Vega and LeTourneau[24] reported that the formation of sclerotia by *Sclerotinia sclerotiorum* was prevented by the omission of zinc ion.

None or quite few sclerotia were formed on the phosphorus absent medium (Table 1). However, as only magnesium was absent, sclerotia of half number and two-thirds weight were formed compared with those on basal medium. Sclerotia were also well formed with some differences when KH_2PO_4 in basal medium was replaced with other phosphates such as K_2HPO_4, $(NH_4)_2HPO_4$ and $CaHPO_4$ (Table 4). The optimal concentration of KH_2PO_4 is in the region of 100 ppm regardless the presence of magnesium (Table 2). For a large number of plants, it has been indicated that the optimal concentration of phosphate in soil is 1–10 μM, whereas 10–20 μM in cytoplasm of plant cell[4,16]. Compared with higher plants, the fungus requires a large amount of phosphate. The number and total weight of the initial and matured sclerotia were increased in proportion to the magnesium concentration (Table 3). Although there was no significant effect of phosphorus on the hyphal linear growth of the fungus, the total weight of the mycelial mat after 2 weeks was obviously excellent at 100 ppm with the presence of magnesium (Table 5). It is suggested that magnesium ion is not always indispensable to the sclerotium morphogenesis but has inductive or promotive effect.

It is widely noted that sufficient growth of hyphae is essential to the subsequent sclerotium formation[5,6]. Wheeler and Sharan[25] showed that the number and weight of sclerotia of *Sclerotium rolfsii* was decreased in proportion to the decrease of KH_2PO_4 concentration but they concluded that phosphate has a lesser effect on the formation than potassium. However, phosphorus is one of the essential element composing nucleic acids which are fundamental factors in living things. If there was no phosphorus at all in medium, no development of hyphae should be observed. A very small amount of phosphorus which allows the hyphal development but not initiates sclerotia might be contaminated into the media as impurities of the ingredients and water used or from air during culture.

In our study on *R. solani*, when KH_2PO_4 in Hopkins medium was replaced with $(NH_4)_2HPO_4$ or $CaHPO_4$, differ in cation, sclerotia were also well produced.

As phosphorus given during the hyphal and initial stages, few sclerotia were formed when the compound was taken away afterwards. Even though the chemicals were omitted during the hyphal and initial stages, good production was recognized when phosphorus was added later (Table 6). These results may indicate that a large quantity of phosphorus is not always necessary for the hyphal development, but indispensable for the sclerotium initiation and subsequent development, enlargement and maturation which required for the high energy production. Thus, no sclerotia may be originated without phosphorus even when ample carbon or nitrogen exist as energy sources. Furthermore, by using isotope (^{32}P), it is indicated that a large amount of phosphorus was accumulated into the sclerotium tissues from media through hyphae (Figs. 2 and 3). Littlefield *et al.*[11] showed that *R. solani* tended to accumulate ^{32}P in sclerotia, hyphal tips, and at branches of hyphae. Mann[12,13] and Bajaj *et al.*[3] elucidated that a large proportion of phosphorus absorbed during the trophophase migrated to the fungal spore in *Aspergillus niger*. Hashiba *et al.*[6,7] proposed that fungal sclerotium is not only the hyphal mass but also the organ which is similar to perithecium and fruit body in morphology and physiology. The fact observed in this experiment could support this proposal and could indicate that phosphorus is, at least, one of the essential constituent of the sclerotium tissue, and shows that the manners of uptake closely resembles to a seed of higher plant and to a fungal spore.

On the other hand, sclerotia were normally initiated and matured without magnesium, though hyphal and matured sclerotium weight increased in proportion to the concentration.

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和 文 摘 要

諸見里善一・石崎文枝・高良このみ・田盛正雄：*Rhizoctonia solani* Kühn (AG-1, IA) の菌核形成に及ぼすリンおよびマグネシウムの影響

基本培地のホプキンス培地から KH_2PO_4 が欠如すると *Rhizoctonia solani* (AG-1, IA) の菌糸は生育したが，菌核はまったく形成されなかった。しかし，$MgSO_4$ が欠如すると菌核数・量とも減少したが，菌核形成は認められた。培地中の KH_2PO_4 を陽イオン部の異なる他のリン酸塩に置き換えた場合，形成数・量に差異は認められたが良好な形成が認められた。KH_2PO_4 を用いたリン酸の最適濃度は菌核形成数・量および菌糸重量ともに 100 ppm 付近であった。マグネシウムを添加すると，菌核数・量ともに濃度に比例して増加した。これらのことからリンイオンは *R. solani* の菌核形成に必須不可欠であり，マグネシウムイオンは誘導促進効果を有することが考えられる。また，菌糸や原基形成の段階までリン酸があっても，それ以降供給がなければ菌核にはわずかしか分化しないのに対し，これらの段階まで供給がなくてもそれ以降の段階に供給されれば対照区と同数・同量の菌核が形成された。リン酸を含まない培地で生育した菌糸の分岐間隔はリン酸を含む培地で生育した菌糸に比べ長い傾向にあった。また，$KH_2{}^{32}PO_4$ を培地に入れ，リン酸の培地から菌体への取り込みとその後の移行を見ると，菌核の形成・成熟期を通してほとんどのリン酸が菌核に移行することが判明した。