

オオムギうどんこ病菌Erysiphe graminis f.sp.hordei発芽胞子の水抽出液はオオムギ子葉鞘の拒否性を高める

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Water-soluble Extract from Germlings of *Erysiphe graminis* Which Enhances Inaccessibility of Barley Coleoptiles*

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Abstract

Water-soluble extracellular materials from germlings of *Erysiphe graminis* f. sp. *hordei* (a pathogen of barley) suppressed infection by the fungus in barley coleoptiles by 23-54% depending on the concentrations applied. The suppressive activity was associated with a low molecular weight (<10,000), heat-stable moiety. The extract preparation was separated into five fractions by HPLC. When applied to lower sides of coleoptiles, only one fraction suppressed infection by *E. graminis* inoculated upper sides of the coleoptiles by about 60%. These suppressive extracts were obtained from conidia and germlings incubated on cellulose membranes 0-12 hr after inoculation, suggesting that conidia of the fungus might constitutively have the elicitor component(s) which could direct barley cells towards the inaccessible state to this fungus.

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Key words : *Erysiphe graminis*, elicitor component, inaccessibility.

INTRODUCTION

In our previous work⁷⁻¹⁴⁾, we studied the accessibility induced by a prior attack of a pathogen, *Erysiphe graminis* f. sp. *hordei* (abbreviated as *E. graminis* below unless otherwise stated), and the inaccessibility enhanced by a prior attack of a nonpathogen, *E. pisi*, at the single cell—single spore level in barley coleoptile cells. When penetration failed in a cell attacked by a nonpathogen, the inaccessible state of the host cell was enhanced leading to subsequent penetration failures by a compatible race of the pathogen^{9,12,13)}. These results led us to assume that penetration attempts by both fungi could be key events triggering physiological changes of host cells towards either the inaccessible or accessible state. However, our later work demonstrated that *E. pisi* released elicitor components at the prepenetration stage which could condition host cells towards the inaccessible state^{11,23)}. Similarly, *E. graminis* appeared to release both elicitor and suppressor components several hours prior to the actual penetration^{7,8)}. In separate studies^{15,16,18)}, we showed evidence that conidia of *E. graminis* secreted esterases, including cutinase, immediately after contact with a host or an artificial membrane. All these studies suggest that components released from *Erysiphe* conidia and/or their germlings might affect host plant cell conditions before the fungus attempts to penetrate the cells.

Cho and Smedegaard-Petersen¹⁾ reported that inocula-

tion of whole barley leaves with either compatible or incompatible races of *E. graminis* resulted in an initial expression of induced resistance to subsequent inoculations even with a compatible race of the fungus. Furthermore, Green *et al.*³⁾ reported that *E. graminis* f. sp. *tritici* elicits an increase in phenylalanine ammonia-lyase (PAL) activity in wheat leaves as early as 4 hr after inoculation. Thereafter, Shiraishi *et al.*^{20,21)} and Clark *et al.*²⁾ confirmed this phenomenon by showing that within 3-4 hr after inoculation of barley leaves with *E. graminis*, the synthesis of cinnamic acid, the immediate product of PAL reaction, was enhanced and that extractable activity of PAL increased by 6 hr and again between 12-15 hr after inoculation. The first elevation of PAL activity occurs during the prepenetration stage, before fungal appressoria mature.

All these previous studies suggested that components which might direct host cells towards the inaccessible state could be released from germlings of *E. graminis* conidia at the prepenetration stage. Therefore, in this study we attempted to extract such substances from *E. graminis* germlings grown on cellulose membranes where initial germling morphogenesis was similar to that occurring on plant surfaces prior to penetration⁶⁾.

MATERIALS AND METHODS

Test plants and fungus Barley (*Hordeum vulgare* L. cv. Kobinkatagi) used as host plant was grown from seed under fluorescent lights (11.8 W m⁻² for 14 hr

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per day). *Erysiphe graminis* de Candolle f. sp. *hordei* Em. Marchal race I was maintained on the host cultivar in a chamber at 20°C, 70% RH (relative humidity) under fluorescent lights (11.8 W m⁻² for 12 hr per day).

Preparation of a water-soluble extract from *E. graminis* germlings Cellulose membranes (11.5 × 3.5 cm²) were prepared from pieces of dialysis tubes (Sanko Jyunkyaku Co.), previously washed in boiling distilled water three times and then dried. Infected leaves with young pustules which had been formed by 6 days after inoculation were used as inocula to avoid inoculation with old conidia. Conidia of *E. graminis* were shaken from infected barley leaves onto the cellulose membranes laid on 2% water agar in a plastic container. The container was kept in a dark chamber conditioned at 20°C and 70% RH. At specified times between 0 and 12 hr after inoculation, five inoculated membranes were removed from the container and shaken gently in distilled water in a petri dish at 2 or 20°C for 5–30 min to obtain a water-soluble extract from the germlings. The germling suspension was filtered through a glass filter (Whatman, GF), then through a millipore filter (Millipore Co., pore size: 0.22 µm), to obtain a germling-free filtrate. The filtrate was lyophilized and stored in a freezer at -80°C. A preliminary test confirmed that shaking the membranes in distilled water did not cause bursting of conidia only when young conidia were used as inocula. The activity of lyophilized material to suppress *E. graminis* infection was examined.

Bioassay of the water-soluble extract Coleoptiles were excised from seedlings 9 days after sowing and partially dissected to prepare single-cell epidermal layers as described previously²². The partially dissected coleoptiles were floated on 0.01 M CaCl₂ solution which had been shown to enhance the infection of *E. graminis*²² and had been used in the bioassay of accessibility and inaccessibility of coleoptile cells in our previous studies^{7–15}.

Lyophilized material was dissolved in distilled water (10 mg/ml) and centrifuged at 4,500 × *g* for 10 min. The supernatant was used as the stock solution of water-soluble extract. Extract solutions ranging from 0.5 to 4 mg/ml were prepared from the stock solution using 0.1 M CaCl₂. The final concentration of CaCl₂ in extract preparations was 0.01 M. The lower sides of coleoptiles were in contact with 50 µl of the respective extract preparations in watch glasses, while the upper sides were inoculated with 50–100 freshly harvested conidia of *E. graminis*. Twenty-four hours after inoculation and incubation at 20°C. Twenty-four hours after inoculation, penetration efficiency (PE: the percentage of appressoria that formed a haustorium) was determined by light microscopy. For a control, inoculated coleoptiles were treated with 0.01 M CaCl₂ solution without the extract, and PE was determined. The activity of the extract in suppressing the *E. graminis* infection was evaluated by comparing PEs in extract-treated and control

coleoptiles using Student's *t*-test.

To examine whether the suppression of PE was due to enhanced inaccessibility in coleoptile cells or to fungitoxicity of the extract preparation, the morphogenesis of *E. graminis* on extract-treated coleoptiles was evaluated. The percentage of primary germ tubes, appressoria, and papilla formation beneath appressoria was determined 24 hr after inoculation.

Treatment of coleoptiles with fractionated or heated extract Three hundred µl of the stock extract solution was separated into fractions of lower and higher molecular weights by ultrafiltration (Millipore Co., LGC24). Lower surfaces of coleoptiles were treated with either fraction for 24 hr after inoculation of upper surfaces with *E. graminis* and PEs were determined.

As described below, the low molecular weight (< 10,000 MW) fraction reduced *E. graminis* infection. Therefore, 20 µl of this fraction was further fractionated by high performance liquid chromatography (HPLC) (Tosoh: column, Tsk-gel PW 3000G). The column was eluted with distilled water at a flow rate of 1 ml/min. The elution profile of the preparation, monitored at 210 nm, is illustrated in Fig. 2. The fractions designated as f₁ through f₅ were collected with a fraction collector. The fractionation was repeated 20 times to obtain enough of each fraction for bioassay. Each fraction was lyophilized, its dry weight determined, then bioassayed as above for activity in suppressing *E. graminis* infection.

The stock extract solution was heated in a water bath at 100°C for 30 min. It was then diluted with distilled water and 0.1 M CaCl₂. Suppression of *E. graminis* infection by this heat-treated extract was bioassayed as described above.

RESULTS

Preparation conditions for a water-soluble extract

On cellulose membranes incubated at 20°C, 21.3% of the conidia produced short primary germ tubes by 1 hr after inoculation, 52.4% by 2 hr. Emergence of appressorial germ tubes was initiated by 4 hr, with about 86% of conidia producing both germ tubes by 6 hr. Most of these germinated conidia produced a mature appressorium with an apical lobe by 12 hr. As confirmed by micromanipulation, such appressoria did not penetrate into the membranes prior to 12 hr, consistent with observations by Kobayashi *et al.*⁶. Thus, we decided that a water-soluble extract should be prepared first from germlings incubated for 12 hr to determine the preparation conditions.

The number of conidia per unit area on each membrane was determined by microscopy. The amount of extract per conidium varied depending on temperature and duration of shaking in distilled water. When membranes were shaken at 20°C and 2°C for 5 min, approxi-

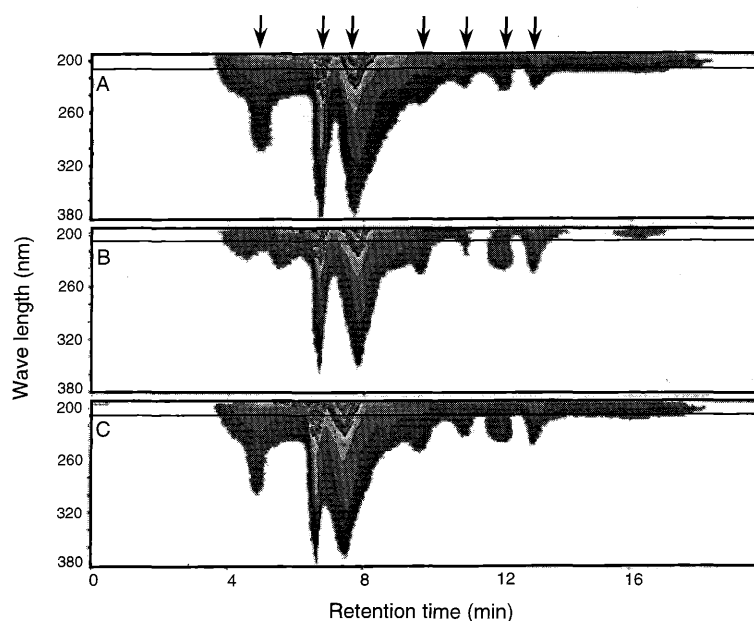


Fig. 1. The HPLC elution profile of the water-soluble extract prepared from *E. graminis* germlings after shaking the cellulose membranes at 20°C for 5 (A), 15 (B) or 30 (C) min from 12 hr after inoculation. Arrows show seven prominent peaks.

mately 0.21 and 0.19 ng extract were obtained per conidium, respectively, whereas 0.43 and 0.42 ng were obtained, respectively, for 15 min. However, the highest amounts were obtained, 1.15 ng at 20°C and 0.60 ng at 2°C for 30 min. Therefore, the fungal extracts were prepared after shaking the cellulose membranes at 20°C for 30 min in the following experiment.

Figure 1-A, B, C demonstrate the 3-D HPLC elution profiles of the extracts which were obtained by shaking inoculated membranes at 20°C for 5, 15 and 30 min in distilled water 12 hr after inoculation, respectively. Based on the retention time, seven prominent fractions were commonly obtained, as arrowed in Fig. 1. Similar fractions were also detected in the extracts which were obtained by shaking the membranes at 2°C, regardless of the shaking times (data are not shown). These results showed that the components of the extract were almost identical, regardless of the temperature and duration of shaking the membranes.

HPLC analyses of water-soluble extracts which were prepared every 3 hr between 0 and 12 hr after inoculation revealed that regardless of the incubation times all the extracts showed similar elution profiles (data are not shown).

Microscopic observations confirmed that 84.9% of the conidium on the membranes had germinated; after washing the membranes by shaking a germination rate of 87.5% was obtained. Furthermore, successive observations of the conidial suspension obtained immediately after immersing the membranes in water revealed that germinating conidia did not burst in water. These observations led us to conclude that the water-soluble extract was released from the outer surfaces of the germlings as opposed to being from inside germlings.

Effects of the water-soluble extract on the E. graminis infection

As shown in Table 1, PE of *E. graminis* averaged 89.3% in coleoptiles which were not treated with the extract (control). When coleoptiles were treated with 2.5–4.0 mg/ml of the extract, PEs were significantly ($p < 0.05$) reduced to 68.0–41.1%. When coleoptiles were treated with only 0.5 and 1.0 mg/ml, PEs were 86.6 and 85.2% which were not significantly different from the control PE.

Subsequently, effects of the 3.0 mg/ml extract on morphogenesis of *E. graminis* in coleoptiles were examined. As indicated in Table 2, morphogenesis, including formation of primary germ tubes and lobed appressoria, subsequent penetration attempts occurred similarly on coleoptiles treated or untreated with the

Table 1. Effects of varied concentrations of the *E. graminis* extract on penetration efficiency of *E. graminis* in coleoptile cells (Lower sides of inoculated coleoptiles were treated with the extract from the onset to the end of incubation)

Concentration of extract (mg/ml)	Penetration efficiency of <i>E. graminis</i> (% $\pm$ SE)
0.5	86.6 $\pm$ 4.2 ^{a)}
1.0	85.2 $\pm$ 2.3 ^{a)}
2.5	68.0 $\pm$ 3.7 ^{b)}
3.0	59.6 $\pm$ 2.8 ^{b)}
4.0	41.1 $\pm$ 3.8 ^{b)}
Control (0.01 M CaCl ₂)	89.3 $\pm$ 5.8

a) No significant difference from PE of control ($p < 0.05$).

b) Significant difference from PE of control ($p < 0.05$).

Table 2. Effects of the *E. graminis* extract on morphogenesis of *E. graminis* on coleoptile cells

Time after inoculation (hr)	Percentage of morphogenesis (%±SE)							
	Primary germ tubes		Appressorial germ tubes		Lobed appressoria		Penetration	
	A ^{a)}	B ^{a)}	A	B	A	B	A	B
3	69.3±4.4	66.1±4.6	0	0	0	0	0	0
6	82.3±3.7	81.5±3.4	41.7±3.3	48.1±2.4	8.7±3.2	11.4±2.2	0	0
8	91.3±1.1	90.8±2.0	79.9±4.6	82.3±5.2	42.9±5.1	50.4±4.6	0	0
11	90.6±1.4	89.6±1.7	91.5±3.2	85.7±4.6	72.1±2.1	76.9±4.1	34.6±4.3	30.5±2.8

a) A: Untreated with the extract (Inoculated Coleoptiles were floated on 0.01 M CaCl₂).

B: Treated with 3.0 mg/ml of the extract.

Table 3. Effects of the fractions of the *E. graminis* extract of a low or high molecular weight on penetration efficiency of *E. graminis* in coleoptile cells

Fraction	Penetration efficiency (%±SE)
Low molecular weight (<10,000)	39.9±3.4 ^{a)}
High molecular weight (>10,000)	86.9±1.1 ^{b)}
Unfractionated	47.8±4.2 ^{a)}
0.01 M CaCl ₂ (control)	85.3±1.9 ^{b)}

a), b) Percentages marked with different letters within a column are significantly different ($p < 0.05$).

extract, showing that fungal morphogenesis up to penetration attempt was not affected by the extract.

All these experiments used the extract prepared from conidia on cellulose membranes after a 12 hr incubation. In separate experiments, extracts prepared from conidia incubated on cellulose membranes for 0–9 hr had suppressive effects on infection similar to those prepared after the 12 hr incubation.

Effects of fractionated and heated extract on *E. graminis* infection

The stock extract solution was separated into two fractions of lower (<10,000) and higher (>10,000) molecular weight. Molecular weight of both fractions was estimated by a gel-filtration standard line drawn using polyethylene glycol (PEG) of known molecular weight. As indicated by Table 3, only low molecular weight fraction suppressed *E. graminis* infection. This fraction was further fractionated by HPLC into five fractions, designated f₁ through f₅ (Fig. 2). The approximate ratios of fraction dry weights were f₁:f₂:f₃:f₄:f₅=5:5:5:1:1. Assuming that the initial crude extract was fractionated into either of these five fractions, 3.0 mg/ml of the crude extract would include 0.88 mg/ml of f_{1–3} each and 0.18 mg/ml of f₄ and f₅ each. Because preliminary tests revealed that *E. graminis* infection in coleoptile cells was suppressed more strongly by low concentrations of f₂ than by any other fractions, inoculated coleoptiles were treated independently with the concentrations shown in Table 4. Only 0.4–0.6 mg/ml of f₂ effectively suppressed infection, showing suppression percentages of 57.9–60.6%. Other fractions were not effective even when high concentrations up to 2.0 mg/ml were applied.

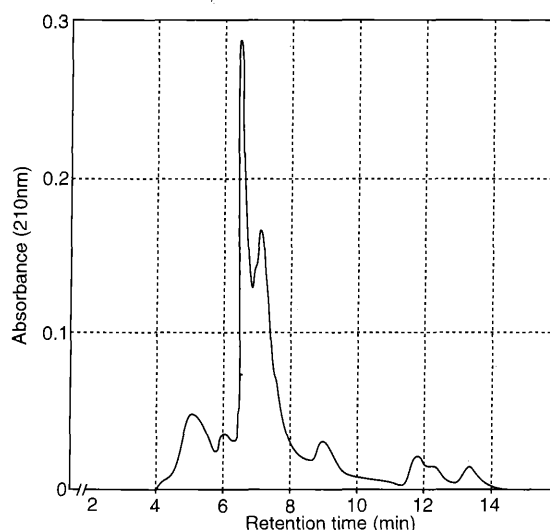


Fig. 2. HPLC elution profile of the fraction of lower molecular weight (<10,000) in the *E. graminis* extract. Five fractions indicated by f₁ through f₅ were collected and used for testing their activity to suppress *E. graminis* infection.

The heated extract did not lose suppressive activity; similar PEs were obtained in coleoptiles treated with either 3 mg/ml of the heated (53.8%) or unheated extract (60.5%). These PEs were significantly lower than that of the control (89.1%) ($p < 0.01$).

DISCUSSION

Kobayashi *et al.*⁷⁾ transferred germlings of *E. graminis* (secondary inoculum) from one coleoptile onto cells of another coleoptile from which the primary germlings of the fungus had been removed with a micromanipulator at various times after inoculation. After an additional 19 hr of incubation, PE of the secondary inoculum was determined. Results showed that inaccessibility was enhanced in the host cells only when the primary inoculum was on the cells for at least 7–8 hr. This period of incubation apparently corresponds to the prepenetration stage, since *E. graminis* had not attempted penetration 10.8±0.2 hr after inoculation under the experimental condition applied in the present study⁷⁾. Their results indicated that *E. graminis* germlings might

Table 4. Effects of fractionated *E. graminis* extract on the *E. graminis* infection in coleoptile cells

Concentration (mg/ml)	Fraction				
	f ₁	f ₂	f ₃	f ₄	f ₅
0.2	—	75.2±7.1 ^{a)}	—	—	—
0.4	—	35.2±4.1 ^{b)}	—	—	—
0.6	—	37.6±3.8 ^{b)}	—	85.7±3.9 ^{a)}	85.7±2.7 ^{a)}
1.0	75.1±5.6 ^{a)}	—	96.0±1.1 ^{a)}	71.0±9.8 ^{a)}	89.9±2.0 ^{a)}
2.0	73.8±8.3 ^{a)}	—	93.7±2.7 ^{a)}	75.6±5.7 ^{a)}	81.2±3.2 ^{a)}

a), b) Percentages including standard error marked with b) are significantly different from that of control (89.3±5.8%) ($p < 0.05$), while those with a) are not significantly different ($p > 0.05$).

release signal(s) that elicited the enhancement of inaccessibility in cells of their barley host plant.

The present results showed that the water-soluble extract of *E. graminis* includes some elicitor component(s) which directed coleoptile cells towards inaccessibility to the fungus. The elicitor factor, estimated to have low molecular weight (<10,000), was heat stable, thus, partially resembling those that Kim and Uritani⁵⁾ separated from mycelial homogenates of *Ceratocystis fimbriata* and that Toyoda *et al.*²³⁾ extracted from germlings of *E. pisi*. The former obtained a water-soluble, dialyzable fraction of low molecular weight that elicited the synthesis of the phytoalexin ipomeamarone in sweet potato, whereas the latter extracted water-soluble fractions of low molecular weight that elicited inaccessibility to *E. graminis* in barley coleoptile cells.

At least one component which probably enhances inaccessibility of coleoptile cells was included in the present extract preparation. Among the concentrations tested, fraction 2 at 0.4–0.6 mg/ml had the greatest suppressive activity against *E. graminis* infection in coleoptile cells, about 60%. Kobayashi *et al.*⁷⁾ incubated conidia from the primary inoculum of *E. graminis* on a single cell of coleoptile (target cell) for 7–8 hr and then removed it by micromanipulation. Subsequently, they transferred a germling of *E. graminis* incubated for 5 hr on a coleoptile onto the target cell of a different coleoptile with a microneedle. PE of the transferred germlings after an additional 19 hr of incubation was suppressed by 44.7–66.7%. This case suggests that the first germlings might release some components which could enhance inaccessibility of the target cell. This degree of suppression was similar to what we obtained with 0.4–0.6 mg/ml of fraction 2. However, the degree of suppression of infection was at most 60–70%, never near 100%. The diversity of physiological conditions of individual coleoptile cells and/or the possible involvement of suppressive factors of *E. graminis*^{7,8)} in extract-treated coleoptiles might be reflected in these results.

As the approximate ratio of fraction dry weight indicated, fractions 4 and 5 were quantitatively minor components in the preparation and could not be tested at higher concentrations. However, further experiments are required to elucidate the effects of higher concentrations of these fractions, as well as possible synergistic effects of the respective fractions.

HPLC profiles of the extracts were similar regardless of the duration of incubation (0–12 hr) of conidia on the membranes. All these extracts showed similar suppressive effects on infection of the present fungus. These results suggest that the putative elicitor(s) may be a constitutive component of conidia and be released during germination.

Examples of the production of both elicitors and suppressors have been shown with fungi such as *Mycosphaerella pinodes*^{17,19)} and *Alternaria alternata*⁴⁾. Although our previous works^{7,8)} demonstrated the presence of suppressor component(s) in *E. graminis* germlings which might prevent enhanced inaccessibility in barley cells, we could not find such factor(s) in our water-soluble preparation. This factor(s) may be water-insoluble and thus not extracted by the present method, the time and amount of release could be limited, or its release may be on the germlings. Further experimentation is required to isolate this factor.

As indicated in Table 2, the extract did not affect morphogenesis of *E. graminis* up to the penetration attempt. This tendency was similar to that observed with the *E. pisi* extract which was applied similarly to barley coleoptiles²³⁾. Since at least 40%, and more than 65% in most cases, of *E. graminis* can produce a haustorium even in the presence of the extract in coleoptiles (Tables 1, 3, 4), we consider that direct interference in haustorium formation by extract is unlikely. However, because we could not examine haustorium formation of *E. graminis* in our *in vitro* system without eliciting host resistance response, we still cannot rule out the possibility that the extract preparation directly suppresses haustorium formation, rather than enhancing inaccessibility of coleoptile cells. Further experiments will be needed to clarify this possibility in some way.

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和 文 摘 要

雪岡日出男・小林一成・久能 均：オオムギうどんこ病菌 *Erysiphe graminis* f. sp. *hordei* 発芽胞子の水抽出液はオオムギ子葉鞘の拒否性を高める

オオムギの病原菌 *Erysiphe graminis* f. sp. *hordei* の発芽胞子から得た水溶性抽出物でオオムギ子葉鞘を処理し、本菌を接種したところ、最大で54%の感染抑制が見られた。その活性は

分子量 10,000 以下の熱耐性成分に依存していた。抽出物を HPLC で分画して得た各分画で子葉鞘の下面を処理し上面に本菌を接種したところ、二番目の画分で感染が約 60%抑制された。本抽出物の溶液に浮かべた子葉鞘上で本菌胞子の形態形成を調べたところ、発芽から侵入に至るまでの過程は阻害されなかった。したがって、本抽出物は子葉鞘細胞の拒否性を高め、

結果的に本菌の感染を抑制する一種のエリシター成分と考えられた。この成分は、セルロース膜上に接種直後の未発芽胞子および同膜上で 3~12 時間培養した発芽胞子から得られたので、本菌の胞子はこの成分を構成的にもっており、発芽に伴って遊離すると推定された。