Mineral Salt Medium for the Growth of *Botrytis cinerea* *in vitro*

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Abstract

A mineral salt (MS) medium suitable for culturing *Botrytis cinerea* *in vitro* is described. The MS medium contains trisodium citrate, MnSO_4 , MgSO_4 , ZnSO_4 , NH_4NO_3 , KH_2PO_4 , CaCl_2 , Na_2MoO_4 , vitamin A palmitate, and sucrose. *B. cinerea* grew vigorously and sporulated numerous conidia in MS agar-medium similarly to those in potato sucrose (PS) agar-medium. The growth was poor if any one of the components in MS medium was omitted. Especially when MgSO_4 , NH_4NO_3 , KH_2PO_4 or sucrose was omitted, almost no growth of the mycelium was resulted, suggesting these components appear to be essential. When cucumber leaves were inoculated with the spores suspended in the MS medium the symptom of gray mold rot appeared, but the symptom did not appear when inoculation was made with spores suspended in distilled water. However, the omission of NH_4NO_3 , KH_2PO_4 or sucrose from the MS medium led to remarkable decrease of infection. A large number of protoplasts with little cell wall debris were isolated by the standard isolation technique⁶⁾ from 40 hr-old mycelium which were cultured in the MS medium. These protoplasts reverted to mycelium at 30-60% after they were incubated in the reversion medium.

(Received November 5, 1986)

Key words : *Botrytis cinerea*, mineral salt medium, protoplast.

Introduction

Botrytis cinerea has been usually cultured *in vitro* on potato-sucrose, potato-dextrose or other media. But the nutrients containing in these media were not defined in any detail, therefore the quality and quantity of the nutrients appear not to be constant from laboratory to laboratory. We think it necessary to establish the chemically defined medium for the biochemical studies on *B. cinerea* such as infection mechanism, mode of action of fungicide, or mechanism of fungicide resistance. MS medium originally came from Murashige and Skoog medium⁵⁾ in which, we noticed, *B. cinerea* grew and sporulated. The aim of the investigation in this report is to determine the components in MS medium necessary for the growth, sporulation and infection of *B. cinerea*.

Materials and Methods

Preparation of *B. cinerea* spores as starting material. A single spore (isolate S-v-5) of *B. cinerea* was grown on potato-sucrose (PS: decoction of 200 g potato and 20 g sucrose in 1 liter of distilled water) agar-medium for 3 days at 22 C. The

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culture was illuminated by near-ultraviolet light (FL 20S, BL-B: Matsushita Co., Ltd.) for 2 days and then sporulated under dark condition for 2 days. The spores formed were washed three times with distilled water and stocked at -80°C .

Measurement of fungal growth in vitro. The spores stocked were suspended in 20 ml of various media to be tested (2×10^4 spores/ml) in Petri dish and incubated for 65 hr at 22°C under the dark. Three replicates were carried out for each medium. The mycelium grown in the Petri dishes was collected and centrifuged at $2,200 \times g$ for 5 min and fresh weight of the precipitate was measured.

Sporulation and spore germination. A droplet ($5 \mu\text{l}$) of distilled water containing 5×10^4 spores was placed on each MS agar (0.5% agarose A45: Nakarai chemicals, Ltd.) -medium in Petri dish and incubated at 22°C under the dark for 4 days. The condition for the sporulation was as described above. The number of spores developed on the agar-medium was counted using a Burkner Turk hemacytometer. Germination of spores was observed in PS medium after incubation for 15 hr.

Inoculation and infection. Leaves of cucumber (*Cucumis sativus* L.) were harvested from 2 week-old plants and punched into disks (20 mm in diameter). These leaf disks were plated on 0.35% agar in Petri dish. Thirty leaf disks, each with one inoculation, were provided for each test medium. Droplets ($10 \mu\text{l}$) of test medium containing 2×10^2 spores were placed on the under leaf-surface, and the disks were kept at 22°C for 4 days to observe the disease development.

Protoplast isolation and reversion. Mycelium cultured in MS medium for 40 hr was used for protoplast isolation. One gram fresh weight of mycelium was suspended in the enzyme solution and shaken for 2 hr at 35°C as previously described⁹⁾. Reversion of protoplasts was performed according to the procedure of thin-layer agar-plate method⁴⁾ with some modifications. Protoplasts were suspended in the reversion medium (MS medium supplemented with 0.54 M mannitol and 1% agarose LGT: Nakarai chemicals, Ltd.) which was maintained at 35°C and solidified to thin layer of 0.1 mm thickness on hemacytometer glass plate and then incubated at 22°C for overnight.

Results

Observation of *B. cinerea* on various MS media in vitro

Components and their concentrations of which we have established for *B. cinerea*, based on Murashige and Skoog medium⁵⁾, were shown in Table 1. The effect of the omission of each component of MS medium on the germination, growth, and sporulation of the fungus was investigated and compared with those in SVg²⁾ (Vogel's mineral salts⁸⁾ with 2% sucrose) or PS medium (Table 2). Germination of spores was remarkably decreased in the medium in which MgSO_4 or KH_2PO_4 was omitted. MgSO_4 , NH_4NO_3 , KH_2PO_4 and sucrose were important components for the growth of *B. cinerea* (Table 2, Plate I, Fig. 1). Trisodium citrate, MnSO_4 , ZnSO_4 , CaCl_2 , Na_2MoO_4 and vitamin A palmitate promoted the growth of mycelium. The microphotographes of *B. cinerea* after 48 hr incubation were shown in Plate I. In the medium without MgSO_4 , the spores germinated but did not grow further, just as in the case of distilled water

Table 1. Components of mineral salt medium for the growth of *Botrytis cinerea* *in vitro*

Components	Concentration (g/liter distilled water)
trisodium citrate	1.5
MnSO ₄ ·4-5H ₂ O	0.5
MgSO ₄ ·7H ₂ O	2.0
ZnSO ₄ ·7H ₂ O	0.001
NH ₄ NO ₃	0.7
KH ₂ PO ₄	4.0
CaCl ₂	0.1
Na ₂ MoO ₄ ·2H ₂ O	0.004
vitamin A palmitate	0.050
sucrose	20.0

Table 2. Effect of the omission of each component from mineral salt medium on the growth and sporulation of *Botrytis cinerea* *in vitro*

Spores suspended in	pH	% spore germination	Growth of mycelium ^{a)}	Spore production ^{b)}
MS medium				
complete	5.25	100	3,141	148
- trisodium citrate	4.42	95.5	2,859	90
- MnSO ₄	5.33	99.3	1,636	8
- MgSO ₄	5.64	65.8	0 ^{c)}	0
- ZnSO ₄	5.26	99.6	1,394	5
- NH ₄ NO ₃	5.26	92.7	43	0
- KH ₂ PO ₄	6.46	62.3	0 ^{c)}	0
- CaCl ₂	5.28	100	1,343	119
- Na ₂ MoO ₄	5.25	99.7	1,691	105
- vitamin A palmitate	5.24	100	2,441	143
- sucrose	5.26	96.8	3	0
PS medium	5.97	99.4	4,823	113
SVg medium	5.85	100	1,101	185
distilled water	—	50.2	0 ^{c)}	0

a) mg fresh weight/20ml medium.

b) × 10⁶ spores/Petri dish.

c) Difficult to collect because of extremely small amount.

(Plate I-3, 8). In the medium omitted NH₄NO₃, the spores germinated and formed repeatedly primary appressorium-like structures (Plate I-4). If KH₂PO₄ was omitted, the spores formed bold germ tubes and then the growth was stopped (Plate I-5). In the medium omitted sucrose, the spores germinated, but developed thin hyphae (Plate I-6). Many secondary appressoria were formed in the medium without trisodium citrate compared with other media (Plate I-2). The appearance of the fungus grown for 2 weeks on agar-media was shown in Fig. 1. The lack of MnSO₄ induced unusual hyphal growth with dark green colour. Sporulation after UV irradiation was extremely poor on the agar-medium which was omitted MnSO₄ or ZnSO₄ (Table 2, Fig. 1). The spores developed on these chemically defined agar-media germinated over 95% when they were

Fig. 1. Effect of the omission of each component in MS agar-medium on the growth of *Botrytis cinerea*. The fungus was cultured for 2 weeks and photographed.

cultured in PS medium.

Infectivity of spores on cucumber leaf

The spores stocked at -80°C were suspended in various test media and inoculated to cucumber leaf disks. The percentage of infection was shown in Table 3. All leaf disks were infected and formed the developing lesions by the spores suspended in PS medium. The spores in MS medium induced similar lesions on about 85% disks with slow lesion development. NH_4NO_3 , KH_2PO_4 and sucrose were important for the lesion development, since infectivity was less than 35% without these components.

Table 3. Infectivity of *Botrytis cinerea* spores suspended in various mineral salt media to cucumber leaves

Spore suspension medium	Infectivity (% of developing lesions)
MS medium complete	85.7
- trisodium citrate	88.9
- MnSO_4	70.0
- MgSO_4	89.3
- ZnSO_4	80.0
- NH_4NO_3	33.3
- KH_2PO_4	20.0
- CaCl_2	81.5
- Na_2MoO_4	92.6
- vitamin A palmitate	90.0
- sucrose	0.0
PS medium	100
distilled water	0.0

Fig. 2. Numerous protoplasts released from 40 hr-old mycelium of *Botrytis cinerea* cultured in MS medium.

Protoplast isolation

Protoplasts from the mycelium which was cultured for 40 hr in MS medium was prepared. A large number of protoplasts (4×10^7 /g f. wt. of mycelium) were isolated and the protoplast preparation was relatively free from the mycelial debris (Fig. 2). Similar results were obtained by repeated experiments. Thirty to 60% of protoplasts were reverted to mycelium when they were incubated in the reversion medium for overnight.

Discussion

From the results described above, MgSO_4 , NH_4NO_3 , KH_2PO_4 and sucrose were essential components for the growth of *B. cinerea*. MnSO_4 and ZnSO_4 promoted sporulation. Trisodium citrate, CaCl_2 , Na_2MoO_4 and vitamin A palmitate promoted the fungal growth. However, *B. cinerea* grew more vigorously in MS medium supplemented with potato extract than in MS medium only (data not shown). Therefore potato extract must contain some other growth factor(s) for *B. cinerea*. Our results presented here support the findings of Harper and Strange²⁾ who reported the effectiveness of glucose, fructose, raffinose, maltose or sucrose as a carbon source for growth of *B. cinerea in vitro* and the fungus was also versatile in its requirement for a nitrogen source (nitrate, ammonium or amino acids) supporting good growth *in vitro* and development of aggressive lesions on broad bean leaves.

Shirane and Watanabe⁷⁾ reported that *B. cinerea* penetrated cucumber leaves not via primary but via secondary appressorium which requires a supply of the exogenous nutrients for its formation. In the MS medium that lacks MgSO_4 , NH_4NO_3 or KH_2PO_4 , the spores could not grow to form secondary appressorium *in vitro*. Thus it was expected that the spores of the fungus suspended in such MS media could not infect cucumber leaf disks. However, the infectivity was not decreased in the medium without MgSO_4 or decreased only to 33.3 and 20.0% in the media without NH_4NO_3 and KH_2PO_4 , respectively. These data indicate that on the surface of cucumber leaves various elements may present; Mg enough for the fungus to infect and N, K, and P also to some extent.

Many investigators demonstrated the infection of *B. cinerea* spores suspended in various media to leaves. For example, Chou and Preece¹⁾ reported that the addition of pollen grains to spore suspension induced the development of spreading lesions on

broad bean leaves. Ko *et al.*³⁾ could accelerate the lesion formation on cucumber leaves by purine related compounds in the presence of glucose. Recently, Harper and Strange²⁾ used SVg medium for the development of lesions on broad bean leaves. Spores can also infect to cucumber leaves by potato extract⁷⁾. These experiments and the data we reported here indicate that *B. cinerea* requires appropriate minerals and carbon and nitrogen sources for the growth and infection to host plant leaves.

The MS medium which we reported here would be useful for various studies on *B. cinerea* *in vitro* and *in vivo* as well as protoplast manipulations, since the medium contains only chemically defined components.

The authors wish to thank Dr. H. Kunoh, Mie University, Mie, and Dr. Y. Hayashi and Mrs K. Hirooka, Shionogi & Co., Ltd., Shiga, for their valuable suggestions and helpful cooperations.

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和 文 摘 要

白根 昇・八田隆行：灰色かび病菌 (*Botrytis cinerea*) 培養用無機塩培地

Botrytis cinerea の *in vitro* 培養に用いるための無機塩培地 (MS 培地) を検討した。MS 培地はクエン酸三ナトリウム, MnSO_4 , MgSO_4 , ZnSO_4 , NH_4NO_3 , KH_2PO_4 , CaCl_2 , Na_2MoO_4 , ビタミンAパルミテート及びシュクロースを含み, 本培地上で菌は旺盛に生育し, 多数の胞子を形成した。又, その生育速度はポテトシュクロース培地に近かった。本培地からいずれの組成を抜いても *B. cinerea* の生育速度は遅れた。特に MgSO_4 , NH_4NO_3 , KH_2PO_4 , シュクロースのいずれかを抜いた場合 *B. cinerea* の生育は著しく低下した。本培地に *B. cinerea* の分生胞子を懸濁し, キュウリ葉に接種すると約85%の発病率 (拡大型病斑) を示した。この培養液から, NH_4NO_3 , KH_2PO_4 , シュクロースのいずれかを抜くと発病率は顕著に低下した。本培養液で培養した菌糸体に酵素処理をすると多量のプロトプラストが分離でき, そのプロトプラストは30~60%の割合で菌糸体に復帰する能力を持っていた。

Explanation of plate

Plate I

Light microscopy of *Botrytis cinerea* which was incubated for 48hr in various MS media (1-6). Compare with PS medium (7) or distilled water (8). Each scale line indicates 10 μm .

Plate I