G. Kritzman and D. Netzer (1978) Phytoparasitica 6(1): 3-7.

A SELECTIVE MEDIUM FOR ISOLATION AND IDENTIFICATION OF *BOTRYTIS* SPP. FROM SOIL AND ONION SEED

By

G. KRITZMAN and D. NETZER*

The population of *Botrytis allii* in either naturally or artificially infested soils was selectively measured by the soil dilution plate count procedure on a developed synthetic medium supplemented with tannic acid, fungicides, antibiotics and Cu^{++}ions. Conversion of tannic acid into a dark brown pigment was related to the activity of extracellular polyphenoloxidase produced by the fungus. Thus, brown-pigmented colonies were recognized as *Botrytis* spp. The same medium was used for detecting the presence of the fungus on onion seed.

KEY WORDS: Botrytis allii; tannic acid; selective inhibition.

INTRODUCTION

Synthetic media which combine selective exclusions with pigment production by the desired fungus have been developed for only a few plant pathogenic fungi. Such media were employed for the isolation and quantitative studies of *Fusarium oxysporum* Schlecht. (2) and *Pyrenochaeta terrestris* (Hans.) Gorenz, J.C. Walker and Larson (7).

Botrytis allii Munn, a common pathogen of onion (*Allium cepa* L.), affects its host mainly during storage. Loss of 50% of onion bulbs due to the neck rot disease was reported (5). Two main sources of primary inoculum have been described. The organism has been reported to overwinter as sclerotia in the soil and in the bulbs (8) and to be seed-borne (5,6).

A selective medium for the assay of *B. allii* from soil was reported by Lorbeer and Tichelaar (3). Although this medium is quite effective in its selective inhibition, identification of the colonies in question as *B. allii* is corroborated only 8-10 days later, on sporulation. While using their medium for detecting seed-borne *B. allii*, the isolation plates were found to be overrun by various fungi.

In the present study a medium for the isolation of *Botrytis* spp. was developed,

Publication of the Agricultural Research Organization. No. 237-E, 1977 series. Received Sept. 20, 1977; received in final form Feb. 28, 1978.

*Div. of Plant Pathology, ARO, The Volcani Center, Bet Dagan.

based on selective inhibition and pigment induction of the organism under study. The latter phenomenon has been treated as a device for rapid identification of the fungus.

MATERIALS AND METHODS

Sporulating cultures of *B. allii* isolated from onion bulbs (cv. 'Ori') were maintained by frequent transfers of single conidia to potato dextrose agar (PDA) slants, which were incubated at 24°C. Standard conidial suspensions were prepared by gently washing the surface of 2-week-old cultures with 20 ml of sterile distilled water containing one drop of Triton x-100.

The basal medium (BM) of the following components (g/l of distilled water) was developed: $NaNO_3$, 1.0; K_2HPO_4, 0.9; $MgSO_4$.$7H_2O$, 0.2; KCl, 0.15; glucose, 20.0; and agar, 25.0. The autoclaved medium was cooled to 70°C, and the other ingredients were added: Terraclor (PCNB), pentachloronitrobenzene 75% WP, 7 x 10^{-3}; Maneb (manganese ethylene bisdithiocarbamate), 4 x 10^{-4}; chloramphenicol, 2.5 x 10^{-2}; $CuSO_4$, 1.7; and tannic acid, 5.0 (Tannin, Sigma Co.). The pH of the supplemented basal medium (SBM) was adjusted to 6.0 with 1N NaOH.

Inoculation technique

Inoculum was grown in 250-ml flasks containing 15 g of wheat bran and 50 ml of tap water, autoclaved twice for 40 min. Three discs (3-mm diam.) of a single spore culture of *B. allii* grown on BM were transferred to each flask. After 7 days' incubation at 24°C the flasks were shaken for 15 min. to ensure uniform distribution of the inoculum; this was followed by an additional 7-days' incubation. The inoculum, composed of mycelium, sclerotia and conidia, was then mixed with a sieved, air-dried, heat-sterilized sandy loam soil. The infested soil was placed in trays (52 x 31 x 10 cm), each of which was planted with 21 onion sets (cv. 'Ori'). The plants were kept for 21 days at 24°C (14 h, day) and 14°C (10 h, night), after which time the final disease assessment was made.

Isolation from soil

Ten g of freshly infested soil was suspended in 100 ml of 0.1% water agar and shaken for 15 minutes. One-ml aliquots of 10^{-3} soil dilution were pipetted into 90-mm diam. petri dishes and spread evenly on the surface of the medium.

Seed test procedure

Commercial onion seed (cv. 'Ori') (60 seeds/plate) was incubated on SBM for 72 h at 24°C.

RESULTS AND DISCUSSION

Addition of tannic acid to culture media provides an easy means of testing for the presence of phenoloxidase in fungi (4). Clark and Lorbeer (1) have reported that *Botrytis cinerea* developed a brown color on catechol-containing medium.

Our selective medium for isolation of *Botrytis* spp. contains tannic acid as a substrate for polyphenoloxidase (PPO) activity. The PPO activity of the growing mycelium of a known culture of *B. allii* was found to convert the tannin in the medium to a dark brown pigment. Several C/N ratios were tested (C as glucose, N as nitrate); a ratio of 20:1 was found to be the most suitable for fast linear mycelial growth, sporulation and pigment development of *B. allii.* Other fungi, such as *Stemphylium* spp. and *Fusarium* spp., which were found by us also to have PPO, were inhibited in our medium by PCNB, Maneb and $CuSO_4$. When medium without glucose was tested, *B. allii* grew well with tannic acid as a carbon source. However, neither PPO activity nor pigment production could be shown under these conditions. Germination of conidia, hyphal elongation and mycelium growth of *B. allii* and *B. cinerea* were not inhibited on SBM.

The SBM medium was utilized for quantitative assay of *B. allii* in artificially infested soil. A positive correlation was found between the increase in inoculum concentrations in soil and the number of brown-pigmented colonies on the dilution plates. Increasing inoculum concentration also increased the percentage of onion sets infected by the fungus (Table 1). Soil samples were collected at harvest time from two fields cropped under onion during the spring of 1976. Five subsamples taken from each field from the top 10-cm soil layer were assayed on the SBM medium. Brown-pigmented colonies could be identified 48 h after incubation at 24°C. At this time, each of the pigmented colonies was transferred to plates with BM. With no exception, all were identified as *B. allii* upon sporulation, after an additional 4 days of incubation.

TABLE 1

INOCULUM DENSITY OF *BOTRYTIS ALLII*, NUMBER OF COLONIES ON SOIL DILUTION PLATES, AND DISEASE INCIDENCE OF ONION (CV. 'ORI') SETS[a]

Inoculum (g/kg soil)	*Colonies/g soil*[b] (±S.E.)	*Disease incidence* (%±S.E.)
0	0	0
0.45	370±5.4	9.5±0.86
0.90	775±8.7	14.2±1.90
1.35	1300±10.3	47.6±3.97

a Twenty-one onion sets were planted in 53 x 31 x 10 cm flats in heat-sterilized sandy loam soil, and kept for 21 days at 24°C (14 h, day) and 14°C (10 h, night) (triplicates).

b Counted on SBM plates after 72 h incubation at 25°C (average of 10 plates/inoculum density).

The results shown in Table 2 indicate that no marked differences were found in the fungal population of the samples taken from the two fields. On the other hand, the

percentage of *B. allii* out of all of the fungal propagules counted on the selective medium was very marked. At Qiryat Gat *B. allii* comprised 35.1% of the population, and at Hebron only 9.9%. The striking difference may well be attributed to the dissimilarity in disease incidence between the bulbs at Qiryat Gat (25%) and at Hebron (traces).

TABLE 2

FUNGAL POPULATION IN SUBSAMPLES OF SOIL FROM TWO FIELDS CROPPED UNDER ONION

Location	*Subsample no.*	*Fungal propagules/g soil*[a] (±S.E.) All	*B. allii*
Qiryat Gat	1	582±18·3	214± 9.1
	2	624±20.1	198± 8.0
	3	630±22.4	232±12.4
	4	590±15.3	221± 7.3
	5	653±17.2	217± 7.5
	Total	2079	1082[b]
Hebron	1	680±19.0	74± 3.0
	2	653±17.8	56± 2.2
	3	674±19.2	81± 4.3
	4	692±21.3	67± 2.6
	5	712±17.2	58± 2.1
	Total	3411	336[c]

a Samples taken at harvest; five plates per sample.
b 35.1% of total population.
c 9.9% of total population.

When onion seed was tested, a brown pigmentation appeared around several seeds after 72 h incubation at 24°C (Fig. 1). Four days later, the brown-pigmented colonies surrounding these seeds were identified as *B. allii.*

In conclusion, colonies obtained on the medium suggested in this paper were brown pigmented and could be identified with ease after 48 h of incubation. Thus, our medium has an advantage over that described by Lorbeer and Tichelaar (3) in that it provides a means for rapid identification and quantitative assay of *Botrytis* spp. from soil and seed.

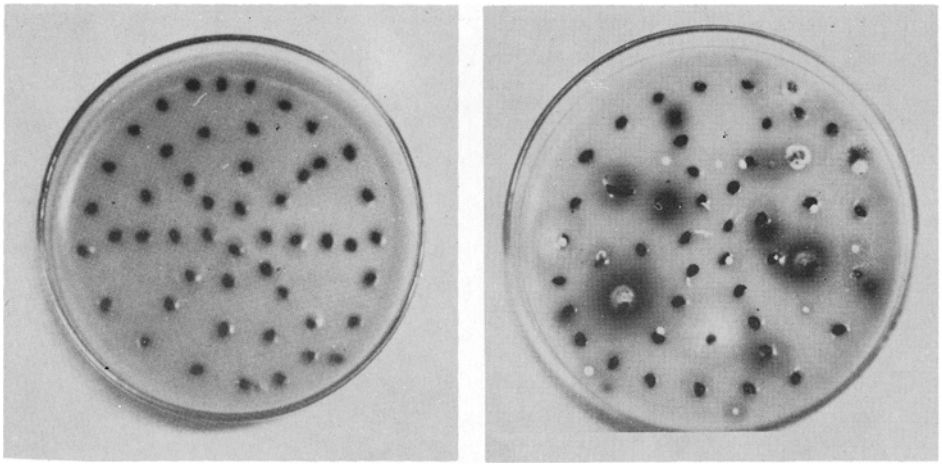

Fig. 1. Commercial onion seed (cv. 'Ori') on SBM medium (left); after 72 h of incubation, brown-pigmented halos surround infected seeds (right).

REFERENCES

1. Clark, C.A. and Lorbeer, J.W. (1975) The role of phenols in Botrytis brown stain of onion. *Phytopathology* **65:** 338-341.
2. Komada, H. (1972) Studies on the method for selective isolation of *Fusarium oxysporum* from natural soil. *Bull. Tokai-Kinki. natn. agric. Exp. Stn* **23:** 144-178.
3. Lorbeer, J.W. and Tichelaar, G.M. (1970) A selective medium for the assay of *Botrytis allii* in organic and mineral soils. *Phytopathology* **60:** 1301 (Abstr.).
4. Lyr, H. (1958) Uber den Nachweis von Oxydasen und Peroxydasen bei hoheren Pilzen und die Bedeutung dieser Enzyme für die Bavendamm Reaktion. *Planta, Berl.* **50**: 359-370.
5. Maude, R.B. and Presly, A.H. (1974) Neck rot of onions. *A. Rep. Nat. Veg. Res. Stn, Wellesbourne* 1973: 94-95.
6. Maude, R.B. and Presly, A.H. (1977) Neck rot (*Botrytis allii*) of bulb onions. 1. Seed-borne infection and its relationship to the disease in the onion crop. *Ann. appl. Biol.* **86:** 163-180.
7. Sneh, B., Netzer, D. and Krikun, J. (1974) Isolation and identification of *Pyrenochaeta terrestris* from soil dilution plates. *Phytopathology* **64:** 275-276.
8. Walker, J.C. (1972) Diseases of Vegetable Crops. McGraw-Hill, New York, N.Y.