Light Microscopic Observation of Nuclei and Mitotic Chromosomes of *Botrytis* Species

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ABSTRACT

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Nuclei and mitotic chromosomes of five species of *Botrytis* that cause diseases in onion were observed with a light microscope. The mean number of nuclei in a conidium of *B. allii* (seven isolates), *B. byssoidea* (three isolates), and *B. cinerea* (five isolates) was 1.3–1.5, 5.0–5.1, and 4.0–5.1, respectively, whereas that of *B. squamosa* (one isolate) was 18.4. The chromosome number was found to be 16 in *B. byssoidea*, *B. cinerea*, *B. squamosa*, and *B. tulipae*. Seven isolates of *B. allii* were divided into two groups according to the chromosome number: three isolates had 32 chromosomes, whereas the other four had 16. In every species used, one of the chromosomes had a threadlike structure. These data indicate that the number of chromosomes in the *Botrytis* species is basically 16 but is 32 in some cases. The species of *B. allii*, *B. squamosa*, and *B. byssoidea* or *B. cinerea* could be distinguished from each other by counting the mean number of nuclei in the conidium.

Additional keyword: conidial dimension.

Botrytis is a fungal genus including 22 species (1) that cause diseases in a wide range of plants. To elucidate the nature of these species, a number of morphological, biochemical, and phytopathological studies have been conducted. However, perhaps because of the small size of the vegetative hyphae, few cytological studies have been done on the nucleus and chromosomes.

Recently, we established a method for observing the mitotic chromosomes of *B. cinerea* and reported that an isolate of the fungus (S-v-5, a single conidium isolate) had 16 chromosomes (5). The present investigation was conducted to determine the chromosome number and observe the nuclear number and dimensions of conidia of five species of *Botrytis* that cause diseases of onion and discuss whether the data can be used for identification purposes.

MATERIALS AND METHODS

Seventeen isolates in five species (seven isolates in *B. allii* Munn, three in *B. byssoidea* Walker, five in *B. cinerea* Pers., one in *B. squamosa* Walker, and one in *B. tulipae* Lind) were used in this study. Details of the isolates are given in Table 1.

Preparation or observation of conidia. Single conidium isolates of *B. cinerea* were grown on potato-sucrose agar (PSA) medium. The isolates were incubated for 3 days at 22 C initially in the dark, exposed 2 days to near-ultraviolet light (FL 20S, BL-B, Matsushita, Osaka), and sporulated under dark conditions for 2 more days. Conidia thus formed were collected and concentrated to 1×10^8 spores/ml in distilled water and stored at −80 C until use. Single conidium isolates of *B. allii* were grown on potato-dextrose agar (PDA) medium. These isolates were incubated for 2 wk at 22 C under dark conditions. Conidia were collected and stored as described above. Isolates of *B. byssoidea* and *B. squamosa* have not sporulated on PSA or PDA but have sporulated on host plant. Therefore, conidia of these isolates were collected from diseased onion leaf and stored at −80 C as described above. Conidia suspended in distilled water were photographed with a light microscope (Microphot-FX, Nikon, Tokyo) fitted with an objective (40×) (PlanApo 40, Nikon). The sizes of 100 conidia in each isolate were measured. The volumes of conidia were calculated as follows:

$$\text{Conidial volume } (\mu m^3) = L \cdot W^2 \pi / 6$$

in which L = conidial length (μm) and W = conidial width (μm). Nuclei in conidia were observed as described previously (5).

Preparation of hyphal fragments. An isolate of *B. tulipae* was used for chromosomal observation. Because the isolate did not sporulate under any cultural conditions examined, the hyphal fragments were used for seed culture. The isolate was grown on PSA at 22 C under dark conditions for 7 days. Mycelia that formed on the medium were scratched with a paintbrush in potato-dextrose broth (PDB). Hyphal fragments suspended in PDB were

TABLE 1. Details of isolates of *Botrytis* species

Species	Isolate no.	Herbarium no.[a]	Host	Sporulation on PDA[b,c]
B. allii	SAL001	···	Onion	++
	SAL002	···	Onion	++
	SAL003	···	Onion	++
	SAL004	IFO9430	Onion	++
	SAL005	HN001	Onion	++
	SAL006	KF297	Onion	++
	SAL007	KF298	Onion	++
B. byssoidea	SAL019	···	Onion	−
	SAL020	···	Onion	−
	SAL021	···	Onion	−
B. cinerea	S-v-18	···	Cucumber	+
	T-v-5	···	Strawberry	+
	T-v-8	···	Eggplant	+
	TI-v-4	···	Tomato	+
	I-t-6	···	Cucumber	+
B. squamosa	SAL022	···	Onion	−
B. tulipae	SAL013	IFO5896	Tulip	−

[a] IFO = Herbarium of Institute for Fermentation Osaka, NH = Herbarium of Hyogo Central Agricultural Experimental Station, and KF = Herbarium of Hokkaido Central Agricultural Experimental Station.
[b] PDA = potato-dextrose agar medium.
[c] − = no sporulation, + = moderate sporulation, and ++ = extremely abundant sporulation.

TABLE 2. Dimensions of measured conidia[a]

Species	Isolate no.	Substrate[b]	Length[a] (μm)	Width (μm)	Length:width ratio	Volume (μm³)
B. allii	SAL001	PDA	10.7 ± 1.5	5.7 ± 0.5	1.9 ± 0.3	185.2 ± 51.5
	SAL002	PDA	10.7 ± 2.1	5.4 ± 0.6	2.0 ± 0.3	171.0 ± 72.9
	SAL003	PDA	8.6 ± 1.1	4.7 ± 0.7	1.9 ± 0.3	103.8 ± 44.1
	SAL004	PDA	10.8 ± 1.3	5.9 ± 0.5	1.8 ± 0.2	200.3 ± 48.2
	SAL005	PDA	8.3 ± 1.1	4.5 ± 0.4	1.8 ± 0.3	89.4 ± 23.6
	SAL006	PDA	8.6 ± 1.2	4.6 ± 0.7	1.9 ± 0.3	99.1 ± 39.5
	SAL007	PDA	8.4 ± 1.2	4.4 ± 0.7	1.9 ± 0.3	88.0 ± 32.8
B. byssoidea	SAL019	Onion	13.0 ± 1.4	8.3 ± 0.9	1.6 ± 0.2	480.6 ± 130.5
	SAL020	Onion	13.4 ± 1.7	8.7 ± 1.1	1.5 ± 0.2	546.7 ± 200.0
	SAL021	Onion	12.7 ± 1.7	8.7 ± 1.0	1.5 ± 0.1	522.6 ± 189.0
B. cinerea	S-v-18	PSA	10.1 ± 1.4	7.6 ± 0.9	1.3 ± 0.2	312.7 ± 90.5
	T-v-5	PSA	10.9 ± 1.4	7.1 ± 0.8	1.5 ± 0.2	292.0 ± 92.4
	T-v-8	PSA	9.5 ± 0.8	6.9 ± 0.6	1.4 ± 0.1	234.0 ± 47.9
	TI-v-4	PSA	10.0 ± 0.8	7.3 ± 0.6	1.4 ± 0.1	285.2 ± 64.5
	I-t-6	PSA	10.4 ± 1.1	7.2 ± 0.7	1.4 ± 0.1	290.1 ± 64.8
B. squamosa	SAL022	Onion	22.7 ± 2.6	15.1 ± 1.6	1.5 ± 0.1	2,804.5 ± 844.5

[a]Average value ± standard error for 100 conidia from each isolate.
[b]PDA = potato-dextrose agar medium, and PSA = potato-sucrose agar medium.

TABLE 3. Numbers of nuclei and chromosomes of *Botrytis* species

Species	Isolate no.	Number of nuclei in conidium[a]	Number of: Chromosomes	Number of: Threadlike structures
B. allii	SAL001	1.5 ± 0.6	32	1
	SAL002	1.4 ± 0.6	32	1
	SAL003	1.4 ± 0.5	16	1
	SAL004	1.5 ± 0.5	32	1
	SAL005	1.3 ± 0.5	16	1
	SAL006	1.3 ± 0.5	16	1
	SAL007	1.4 ± 0.5	16	1
B. byssoidea	SAL019	5.0 ± 1.4	16	1
	SAL020	5.1 ± 1.4	16	1
	SAL021	5.1 ± 1.8	16	1
B. cinerea	S-v-18	5.1 ± 1.4	16	1
	T-v-5	4.2 ± 1.1	16	1
	T-v-8	4.0 ± 1.4	16	1
	TI-v-4	4.9 ± 1.2	16	1
	I-t-6	4.6 ± 1.3	16	1
B. squamosa	SAL022	18.4 ± 4.4	16	1
B. tulipae	SAL013	Not tested	16	1

[a]Average value ± standard error for 100 conidia from each isolate.

collected and used.

Observation of chromosomes. Conidia or hyphal fragments suspended in PDB were incubated on glass slides at 22 C for various periods (6.5 hr for *B. cinerea*, 8–9 hr for *B. byssoidea* and *B. squamosa*, and 9–17 hr for *B. allii* and *B. tulipae*), and the germlings and hyphal fragments were treated with methanol-acetic acid solution (methanol:acetic acid = 17:3, v/v) for 30 min and flame dried. The specimens were transferred to 95% ethanol and kept in 70% ethanol for 3 hr. After hydrolyzation in 1 N HCl for 5 min at room temperature and for 10 min at 60 C, they were washed with distilled water and stained with Giemsa (Giemsa's Losung, Merck, Darmstadt) at 3.5%, v/v, in 1/15 M phosphate buffer, pH 7.0, for 3 hr. After being rinsed in tap water, they were air dried and observed with a light microscope (Microphot-FX) fitted with an objective lens (100×) (PlanApo 100, Nikon).

RESULTS AND DISCUSSION

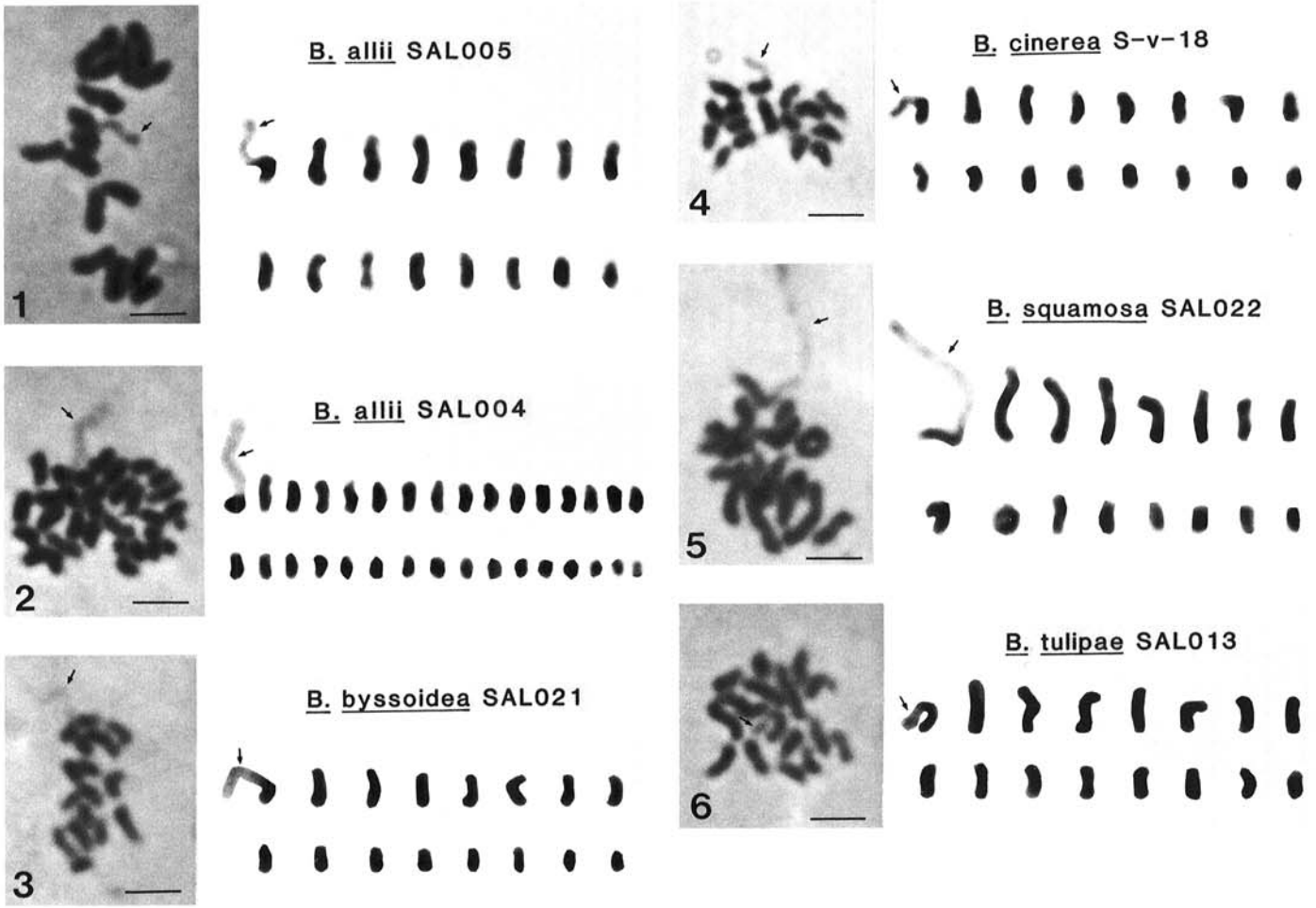
Conidial dimensions. Conidial dimensions differed among the four species studied (Table 2). The mean sizes of three isolates of *B. byssoidea* were found to be 12–14 × 8–9 μm, and the mean volumes were 480–547 μm³. Five isolates of *B. cinerea* were 9–11 × 6–8 μm (volume, 234–313 μm³). An isolate of *B. squamosa* was 23 × 15 μm (volume, 2,805 μm³). Seven isolates of *B. allii* were separated into two groups: one including SAL001, SAL002, and SAL004, with a mean size of 10–11 × 5–6 μm (volume, 170–200 μm³), and the other including SAL003, SAL005, SAL006, and SAL007, with a mean size of 8–9 × 4–5 μm (volume, 88–104 μm³). The mean ratios of the length to the width of the conidia were 1.3–1.6 in *B. byssoidea*, *B. cinerea*, and *B. squamosa*. On the other hand, the mean ratios were 1.8–2.0 in *B. allii*. Conidial dimensions of length and width may be used to distinguish *B. allii*, *B. byssoidea*, *B. cinerea*, and *B. squamosa* from each other.

Walker (6) stated in his review that the conidia of *B. allii* are oblong to elliptical in shape and that the majority of the conidia fall within the range of 7–11 × 5–6 μm. In this study, almost all mean sizes of two groups in *B. allii* fall within the range described by Walker.

Number of nuclei in a conidium. Nuclei were observed for *B. allii*, *B. byssoidea*, *B. cinerea*, and *B. squamosa*. The number of nuclei in conidia of *B. cinerea* was similar to that of *B. byssoidea*. The mean numbers for the two species were 4.0–5.1. The means of *B. allii* and *B. squamosa* were 1.3–1.5 and 18.4, respectively (Table 3). Thus, the mean number might be used for taxonomic study. Phillips et al (4) and Lorbeer (2) reported that cultures of the same isolate of *B. cinerea* growing on various media differ in the size and nuclear number of conidia. In the present study, the substrate used to produce the conidia was not equal and could have influenced the results. To identify *Botrytis* species precisely, consistent conditions for the sporulation of the conidia must be established.

Several investigators (2,3) have suggested that *B. allii* and *B. byssoidea* are conspecific because they are very similar except for sporulation on culture media and a relatively small difference in the range of length and width of conidia. The present results indicate that *B. allii* is unique in several distinctive features if compared with other species. Conidia of *B. allii* were narrowly ellipsoidal and distinctly narrower than those of other species (Table 2), and most of the conidia included only one or two nuclei (Table 3). *B. allii* formed a much greater number of conidia on artificial media than other species (Table 1). These data suggest that *B. allii* and *B. byssoidea* are not conspecific.

Microscopic observation of chromosomes. The number of mitotic chromosomes in each isolate is shown in Table 3, and Figures 1 through 6 are enlarged photographs of chromosomes of each species. All five isolates of *B. cinerea* that were collected from different fields and at different periods had 16 chromosomes. Therefore, the number reported previously for isolate S-v-5 (5) may be basic for this species. All isolates of *B. byssoidea*, *B. squamosa*, and *B. tulipae* had 16 chromosomes, and one of the



Figs. 1–6. Enlarged photographs of the chromosomes of *Botrytis* species at metaphase. The chromosomes are arranged in descending order of length. Arrows indicate the threadlike structure. Bar = 2 μm.

chromosomes had a threadlike structure (TLS).

The seven isolates of *B. allii* could be divided into two groups. One of them had 32 chromosomes and the conidial volumes were 171–200 μm^3 on the average (SAL001, SAL002, and SAL004). The other group (SAL003 and SAL005–SAL007) had 16 chromosomes and the mean conidial volumes were 88–104 μm^3. On the other hand, the mean ratio of the length to the width of conidia, and the number of nuclei in a conidium were similar between the two groups (Tables 2 and 3). These findings indicate that the chromosome number is correlated with the volume of the conidium and suggest that the isolates that have 16 chromosomes may be haploid and those that have 32 chromosomes may be diploid. In the 32-chromosome isolates, however, only one chromosome was associated with TLS. We have no explanation for this and also cannot deny the possibility of overlapping the faint TLS with chromosomes. If the 32-chromosome isolate could be produced from the 16-chromosome isolate by artificial mutation, this question would be resolved.

In summary, we found that the number of chromosomes in the *Botrytis* species is basically 16 but is 32 in some cases. *B. allii* exists as two different isolates in the field, one having 16 chromosomes and the other 32. The number of mitotic chromosomes could not be used to distinguish among the five species examined. However, the number of nuclei in the conidium and the conidial dimension should be useful criteria for identifying *Botrytis* species.

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