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Variation in growth at different temperatures and production and size of conidia in hybrid strains of *Verticillium lecanii* (*Lecanicillium* spp.) (Deuteromycotina: Hyphomycetes)

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Abstract

In a previous study, we confirmed alterations in several fungal traits in hybrid strains of *Verticillium lecanii* generated via protoplast fusion. In the present study, we surveyed radial colony growth in different temperature regimes as well as conidia productivity and conidia size of hybrid strains of *V. lecanii* to elucidate the various primary fungal properties of these strains and to determine the correlation between these properties and virulence to insect pests. Minimum temperature thresholds for all tested strains (except Vertalec®) were below 5°C. Optimal temperatures for colony growth were 20–25°C. Colony growth at high temperature was good or poor depending on the genotype. Temperature and strain significantly affected colony growth. Conidial production and conidia size were also significantly affected by strain. Conidial production differed by almost 170 times between the highest conidia-producing strain BbF10 and the lowest conidia-producing strain AaF30, although both strains originated from the same parents. Hybrid strains tended to show slightly larger conidia than their parent strains of the same genotype. Although all these traits varied considerably among hybrid strains, no apparent relationship of these traits with virulence to insect pests was found.

Key words: *Lecanicillium* spp.; phenotypic variation; protoplast fusion; temperature growth response; *Verticillium lecanii*

INTRODUCTION

Verticillium lecanii (Zimm.) Viégas (*Lecanicillium* spp.) is a well-known entomopathogenic fungus. It has an extremely wide host range that includes aphid and scale (Hall, 1981), whitefly (Hall, 1982), phytopathogenic fungi (Verhaar et al., 1998; Spencer and Atkey, 1981), and plant parasitic nematodes (Meyer and Wergin, 1998; Eapen et al., 2005; Shinya et al., 2008). Highly virulent and epizootically efficient strains of *V. lecanii* have been mass-produced as biocontrol agents against some insect pests. Vertalec® and Mycotal® (Koppert Biological Systems, Netherlands) are commercial formulations of developed *V. lecanii* strains and are recommended for application against greenhouse aphids, whitefly and thrips, respectively. *Verticillium* section *Prostrata* was re-classified in the genera *Lecanicillium*, *Pochonia*, *Haptocillium*, and *Simplicillium* based on morphological observations and molecular analysis (Zare et al., 2000; Gams and Zare, 2001; Sung et al., 2001; Zare and Gams, 2001). The genus *Lecanicillium* includes 15 species; Vertalec® belong to *L. longisporum*, and Mycotal® and B-2 (described later) belong to *L. muscarium* (Zare and Gams, 2001; Sugimoto et al., 2003; Koike et al., 2007).

The processes of conidial germination and

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growth of most entomopathogenic fungi are highly dependent on both available moisture and temperature. In fact, *V. lecanii* principally requires high humidity to kill insect pests (Ekbom, 1979; Hsiao et al., 1992). Consequently, the application of *V. lecanii* has remained limited to greenhouses. Recently, Koike et al. (2004) isolated the B-2 strain (MAFF238429) from green peach aphid in Obihiro; this strain has high viability on living plant leaf surfaces under low humidity conditions. Subsequently, Aiuchi et al. (2008) conducted a protoplast fusion study with 3 strains (Vertalec®, Mycotal®, and B-2) of *V. lecanii* using the polyethylene glycol method and a nitrate non-utilizing mutant as a genetic marker. A total of 126 hybrid strains from Vertalec®×Mycotal® (AaF fusant group (FG: assemblies of hybrid strains derived from the same parents) and BbF-FG), 4 hybrid strains from Vertalec®×B-2 (2BF-FG), and 44 hybrid strains from Mycotal®×B-2 (2aF-FG) were produced via protoplast fusion.

Many studies have reported fungal protoplast fusion. Most of these studies specifically describe the techniques of protoplast fusion, fungal genetics, or strain improvement of industrially important fungi (Lalithakumari, 2000); however, despite the availability of a variety of hybrid strains, little attention has been given to primary fungal properties of these strains such as those described in the present study.

V. lecanii has no resting structures similar to those found in other *Verticillium* spp.; for example, dictyochlamydospore in *V. chlamydosporium* (*P. chlamydosporia*) and *V. suchlasporium* (*P. suchlasporia*) (Zare et al., 2001), microsclerotia in *V. dahliae*, and dark resting mycelium in *V. albo-atrum*. Furthermore, the upper limit of temperature for mycelial growth of *V. lecanii* is <31°C to 34°C (11 of 16 strains showed growth at 31°C; 4 of 16 strains at 34°C; and only 1 of 16 strains showed slight growth at 36°C) (Hall, 1981). This upper limit of temperature is lower than those of other entomopathogenic fungi such as *Beauveria bassiana* (35–37°C; Fargues et al., 1997) and *Metarhizium anisopliae* (40°C; Hallsworth and Magan, 1999). A wider temperature range for fungal growth is advantageous for use as a biological control agent because the temperature of an uncontrolled greenhouse usually increases above 35°C in summer.

In our previous study, in which the banding pattern of fragmented DNA of *V. lecanii* was determined in genomic DNA analysis by arbitrarily primed-polymerase chain reaction (AP-PCR), we found that *V. lecanii* showed alterations in colony appearance and virulence against insect pests (Aiuchi et al., 2007, 2008); therefore, we also expected alterations in other fungal traits of *V. lecanii*. In the present study, we measured radial colony growth, conidia productivity, and conidia size of hybrid strains to elucidate the various fungal traits of hybrid strains and to determine the correlation between these traits and virulence to insect pests.

MATERIALS AND METHODS

Fungal strains. Strains of *V. lecanii* used in the radial colony growth study and the survey of conidiation ability included 3 parental strains (Vertalec®, Mycotal®, and B-2) and 82 hybrid strains (44 strains from 2aF-FG, 4 strains from 2BF-FG, 25 strains from AaF-FG, and 9 strains from BbF-FG). Strains of *V. lecanii* used in the conidial size study included 3 parental strains and 159 hybrid strains (91 strains from AaF-FG, 20 strains from BbF-FG, 44 strains from 2aF-FG, and 4 strains from 2BF-FG) (Aiuchi et al., 2008). Vertalec® and Mycotal® were single spores isolated from the formulations.

Effects of temperature on radial colony growth. All 83 strains of *V. lecanii* were cultured on potato dextrose agar (PDA; Difco Laboratories) for 10 d at 24°C in the dark. Each mycelial disk was taken using a flame-sterilized cork borer (5 mm diameter). The disk was then placed upside down at the center of a fresh PDA plate (90 mm diameter) with one mycelial plug per plate. We used 7 constant temperature regimes: 5, 10, 15, 20, 25, 30, and 35°C. Strains used in the radial colony growth test at 35°C included 3 parental strains and 7 hybrid strains (5 and 2 strains from 2aF-FG and 2BF-FG, respectively, showing good growth at 30°C). Radial colony growth was recorded weekly (up to 21 d) using a digital caliper by measuring the diameter for every 120° (3 directions) of the fungal colony from the bottom of each plate. These colony diameter measurements were performed in 5 replications per strain and temperature.

Measurement of conidia production ability. To measure conidiation, each mycelial disk was

taken using a cork borer (10 mm diameter) from the edge of a 21-d-old colony on a PDA plate (Difco Laboratories) incubated at 25°C and suspended in 10 ml of distilled water (one mycelial disk per plate). Conidial concentration was determined using a hemocytometer under a light microscope. These measurements were conducted in 5 replications.

Conidial size. Fungal strains used in this study were cultured on a PDA plate (90 mm diameter; Difco Laboratories) for 11 d at 25°C in the dark. Conidia were then harvested by flooding the plate with distilled water; the length and width of 50 conidia were measured using a micrometer under a light microscope.

Data analysis. Differences in the colony sizes of the tested strains at each temperature and conidial production, conidial length, and conidial width of the tested strains were compared using one-way and two-way analyses of variance (ANOVAs) with Tukey's honestly significantly different (HSD) test (α=0.01). Differences in the colony size, conidial production, conidial length, and conidial width of parental strains and each FG were compared using one-way ANOVA with Scheffe's F test (α=0.01).

RESULTS AND DISCUSSION

The peak radial colony growth of BbF-FG occurred at 20°C; AaF-FG, at 20°C (13 of 25 strains) or 25°C (12 of 25 strains); and Vertalec®, Mycotal®, B-2, 2aF-FG (except 2aF42), and 2BF-FG, at 25°C (Fig. 1). No strain showed growth at 35°C. The maximum temperature threshold of all tested strains was higher than 30°C, except for Vertalec® (Table 1). The minimum temperature threshold of all tested strains was below 5°C, except for Vertalec® (Table 1). The maximum and minimum temperature thresholds of Vertalec® were not similar to those reported by Kope et al. (2008) and Hall (1981). This might have been due to a difference in the medium used and/or lot number of *V. lecanii* formulations. Radial growth was significantly influenced by the strain ($F_{85,1032}$=187.71; p<0.01) and temperature ($F_{5,1032}$=94500.54; p<0.01); an interaction between strain and temperature ($F_{425,1032}$=110.68; p<0.01) was also found.

Some hybrid strains had a colony diameter larger than that of their parental strains at each temperature, and the growth of hybrid strains exhibited overall improvement (Table 1). AaF6 and AaF8 showed markedly faster growth than their parental strains; however, no remarkable difference was found in the growth of other hybrid strains at 5°C (Table 1). The BbF-FG colony particularly showed fast growth at the suboptimal temperature (10, 15, and 20°C; Table 1, Fig. 1). On the other hand, at higher temperatures of 25 and 30°C, 2aF-FG and 2BF-FG showed faster radial colony growth (Table 1, Fig. 1). A notable feature of BbF-FG strains was

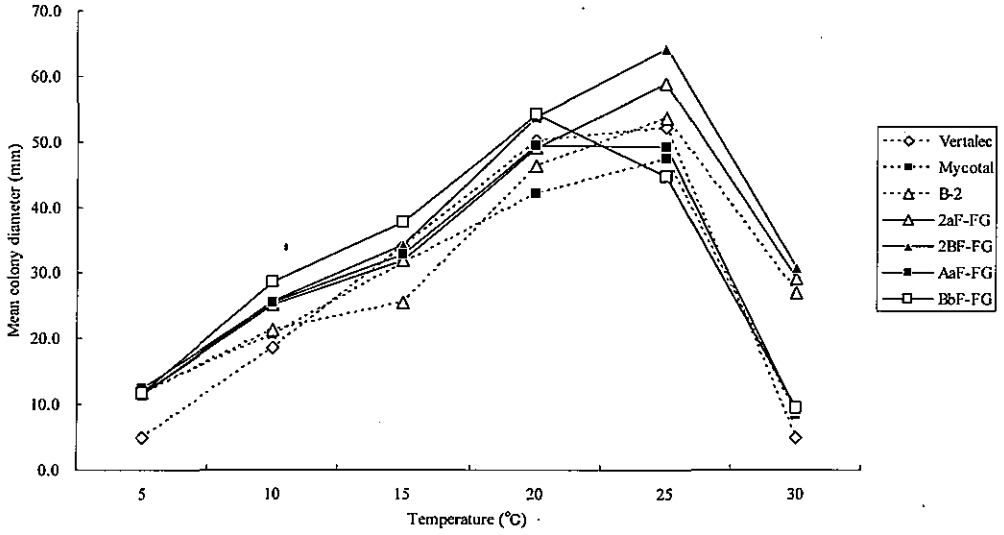

Fig. 1. The mean colony diameter of parental strains and fusant groups of *Verticillium lecanii* in different temperature regimes. Exact values are presented in Table 1.

Table 1. Temperature susceptibility of radial colony growth and conidia production of *Verticillium lecanii* hybrid strains and parental strains

Strains	Colony size (diameter: mm)																		No. of conidia ($\times 10^6$/cm^2)		
	5°C			10°C			15°C			20°C			25°C			30°C					
	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
Parental strains																					
Vertalec	5.0	0.0	C[a], x[b]	18.6	0.3	D, n′	34.1	0.2	BC, f-n	50.2	0.5	AB, k-x	52.1	0.6	BC, p-u	5.0	0.0	B, w	16.3	1.6	B, d
Mycotal	12.1	0.1	A, c-m	20.5	0.1	CD, l′-m′	31.5	0.3	CD, s-b′	42.1	0.3	C, f′	47.3	1.0	CD, x-a′	8.3	0.1	B, u-w	48.6	2.4	AB, cd
B-2	11.6	0.1	AB, e-s	21.3	0.2	C, k′-l′	25.4	0.2	D, e′	46.2	0.8	BC, b′-e′	53.6	0.6	BC, o-t	26.9	0.2	A, l-s	69.9	10	AB, c
2aF-FG (Mycotal×B-2)																					
2aF1	12.1	0.1	b-m	27.5	0.1	e-j	30.3	0.5	y-c′	47.4	0.4	w-d′	54.5	1.6	l-q	26.4	0.2	o-s	18.9	4.2	d
2aF2	13.4	0.1	a-d	26.7	0.1	f-q	38.3	0.5	b-d	50.0	1.7	k-y	63.0	1.8	e-g	41.3	0.5	a	25.0	6.1	d
2aF3	12.1	0.0	d-o	27.6	0.2	d-h	33.0	0.2	h-v	52.9	0.2	e-m	60.9	1.4	f-h	25.6	1.4	q-s	73.9	14	bc
2aF4	11.6	0.0	e-s	26.2	0.1	h-w	32.0	0.3	l-z	51.2	0.4	j-t	56.5	0.7	i-o	21.6	1.5	t	95.1	5.7	bc
2aF5	11.5	0.2	f-s	25.8	0.3	k-b′	31.4	0.2	s-c′	47.1	1.2	x-d′	58.1	0.8	h-k	31.2	0.7	g-j	16.8	2.1	d
2aF6	11.3	0.6	i-t	27.1	0.3	f-m	32.5	0.3	i-y	54.5	0.6	c-h	66.3	0.2	c-e	26.5	0.4	m-s	42.7	1.1	cd
2aF7	11.7	0.4	e-r	26.5	0.3	f-s	31.9	0.6	m-z	47.1	0.5	x-d′	55.3	0.6	k-p	29.5	0.3	i-o	23.3	9.1	d
2aF8	10.6	0.0	p-w	26.3	0.3	g-t	31.8	0.7	o-z	47.1	0.1	x-d′	56.8	0.1	i-o	27.7	0.4	k-s	12.1	1.4	d
2aF9	10.4	0.1	q-w	27.2	0.3	e-l	29.2	1.2	c′-d′	48.0	0.8	t-d′	57.1	1.0	i-n	27.7	0.2	k-s	15.2	2.1	d
2aF10	11.4	0.1	h-t	25.7	0.2	m-b′	31.8	0.4	o-z	46.3	0.2	a′-e′	58.1	0.2	h-k	28.3	0.5	j-r	16.2	1.2	d
2aF11	11.7	0.1	e-r	25.9	0.3	k-a′	32.5	0.3	j-y	48.5	0.6	q-d′	58.1	1.6	h-k	31.6	2.2	f-j	12.3	1.4	d
2aF12	11.9	0.2	d-p	23.8	0.3	e′-j′	29.5	0.2	a′-d′	49.2	0.3	o-b′	57.9	0.3	h-l	29.9	1.0	h-l	36.2	2.6	cd
2aF13	10.4	0.5	r-w	24.8	0.3	u-i′	32.1	0.5	l-z	50.9	0.2	j-u	66.8	1.6	b-d	27.4	0.3	k-s	35.0	5.9	cd
2aF14	9.4	0.3	v-w	23.9	0.1	e′-j′	30.5	0.4	x-c′	46.0	0.5	c′-e′	59.4	0.1	h-j	25.5	0.9	r-s	32.5	3.4	cd
2aF15	11.9	0.2	d-p	26.6	0.1	f-r	31.7	0.2	q-a′	48.2	0.3	r-d′	57.9	0.2	h-l	27.6	0.5	k-s	31.1	1.1	cd
2aF16	11.8	0.0	d-p	25.8	0.1	l-b′	34.1	0.1	f-o	54.7	0.3	c-h	66.9	0.5	b-c	34.8	1.4	c-f	24.0	2.1	d
2aF17	12.1	0.4	b-l	26.2	0.3	g-v	34.8	0.1	e-i	51.3	0.8	i-r	64.8	0.4	c-e	29.7	0.2	h-m	31.3	4.5	cd
2aF18	10.3	0.1	s-w	25.6	0.2	n-b′	32.0	0.3	l-z	47.3	0.1	w-d′	57.3	0.7	i-n	27.6	0.4	k-s	16.1	3.6	d
2aF19	12.0	0.1	d-o	23.4	0.3	i′-j′	31.5	0.3	r-b′	50.5	1.3	k-w	58.5	1.2	h-k	24.8	0.6	s-t	16.4	2.1	d
2aF20	10.8	0.1	m-v	23.5	0.4	i′-j′	31.3	0.5	s-c′	46.5	0.5	a′-e′	57.0	0.3	i-o	27.4	0.5	k-s	8.6	0.8	d
2aF21	11.9	0.1	d-p	24.0	0.2	e′-i′	31.1	0.8	u-c′	50.0	0.3	k-y	58.6	0.4	h-k	27.4	0.2	k-s	7.3	2.9	d
2aF22	10.1	0.2	t-w	22.4	0.1	j′-k′	27.3	0.6	d′-e′	46.4	1.0	a′-e′	55.6	1.1	k-o	33.9	0.4	d-g	11.2	0.6	d
2aF23	9.6	0.2	u-w	21.6	0.4	k′-l′	29.3	0.7	b′-d′	42.1	0.2	f′	53.9	0.4	n-s	27.8	0.4	k-s	12.0	0.5	d
2aF24	11.8	0.4	e-q	26.9	0.3	f-o	33.0	0.5	h-w	50.2	1.0	k-x	58.6	1.7	h-k	28.3	1.3	j-r	11.6	2.1	d
2aF25	11.2	0.3	i-t	23.9	0.1	e′-i′	31.3	0.4	t-c′	46.9	0.5	y-d′	56.5	0.1	i-o	26.2	0.5	p-s	23.4	1.1	d
2aF26	11.4	0.3	g-t	24.5	0.4	a′-i′	30.7	0.2	w-c′	46.8	0.5	z-d′	51.4	0.4	q-v	24.5	0.2	s-t	8.6	0.6	d
2aF27	10.8	0.1	l-u	24.6	0.3	y-i′	31.1	0.5	v-c′	45.3	0.2	d′-e′	57.7	0.4	h-m	29.2	0.3	i-p	17.1	1.5	d
2aF28	11.6	0.1	e-s	25.9	0.1	k-z	31.8	0.4	p-z	47.6	0.2	v-d′	56.5	0.3	i-o	27.2	0.4	k-s	38.0	2.1	cd
2aF29	10.9	0.3	l-u	26.3	0.1	g-u	36.3	0.1	d-f	53.1	1.2	d-k	58.0	0.3	h-l	30.1	2.9	h-l	7.6	0.4	d
2aF30	11.3	0.1	i-t	24.6	0.1	y-i′	31.2	0.8	t-c′	47.4	0.0	w-d′	57.8	0.2	h-l	31.7	0.5	f-i	8.9	1	d

2aF31	11.1	0.0	j-t	23.7	0.6	g′-j′	31.5	0.7	s-b′	50.8	0.6	j-v	57.5	0.6	h-m	26.9	0.6	l-s	14.2	4.8	d
2aF32	11.4	0.0	f-s	25.5	0.1	o-d′	32.6	0.1	i-y	53.7	0.1	c-j	65.5	0.1	c-e	32.9	0.2	e-h	27.7	5.6	d
2aF33	11.3	0.2	i-t	24.1	0.2	c′-i′	32.1	0.3	l-z	47.6	0.1	v-d′	57.7	0.6	h-l	30.4	0.1	h-k	13.7	1.5	d
2aF34	11.8	0.1	d-p	25.1	0.1	s-g′	34.4	0.3	f-k	49.9	0.2	l-z	59.8	0.2	g-i	25.4	0.5	r-s	2.5	0.7	d
2aF35	12.3	0.1	b-k	24.5	0.3	z-i′	34.1	0.2	f-m	55.9	0.3	b-e	66.8	0.2	b-d	38.6	1.0	a-b	28.5	0.4	d
2aF36	11.9	0.0	d-p	24.4	0.0	b′-i′	32.2	0.5	k-y	49.5	0.0	n-a′	58.0	0.2	h-k	38.8	1.0	a-b	13.7	1.8	d
2aF37	12.1	0.3	d-o	24.8	0.3	v-i′	31.6	0.3	q-b′	48.0	0.4	u-d′	57.4	0.0	h-m	37.5	0.2	b-c	15.5	3.4	d
2aF38	11.9	0.2	d-p	23.5	0.4	h′-j′	29.9	0.3	z-c′	48.4	0.2	r-d′	57.7	0.5	h-l	37.1	0.8	b-d	12.0	1.6	d
2aF39	11.8	0.1	d-p	24.4	0.1	b′-i′	31.9	0.2	m-z	48.5	0.1	q-d′	56.2	0.1	j-o	28.9	0.3	i-q	14.7	1.1	d
2aF40	12.8	0.2	a-f	24.6	0.3	y-i′	34.0	0.7	f-p	46.1	0.5	b′-e′	63.4	0.1	d-f	37.1	0.5	b-d	9.8	1.6	d
2aF41	12.0	0.1	d-o	25.6	0.4	n-b′	31.6	0.7	q-b′	46.3	0.8	b′-e′	58.7	0.3	h-k	26.8	0.1	l-s	7.3	0.4	d
2aF42	10.7	0.1	o-w	24.6	0.1	y-i′	25.5	1.1	e′	60.0	0.1	a	55.4	0.3	k-p	9.2	0.4	u-v	2.4	0.4	d
2aF43	10.7	0.1	n-w	23.8	0.5	g′-j′	31.6	0.1	q-a′	46.4	0.6	a′-e′	59.3	0.4	h-j	26.4	0.6	m-s	50.8	2.8	cd
2aF44	11.4	0.3	h-t	24.1	0.5	c′-i′	32.0	0.3	l-z	48.2	0.2	r-d′	58.7	0.5	h-k	26.0	0.6	p-s	9.8	0.8	d
Mean	11.4	0.07	B	25.1	0.12	B	31.8	0.19	C	49.1	0.29	B	58.8	0.32	B	29.1	0.47	A	21.6		B
2BF-FG (Vertalec×B-2)																					
2BF1	10.9	0.2	l-u	26.0	0.2	k-y	31.8	0.1	n-z	55.3	0.5	b-f	72.4	0.2	a	35.1	0.3	c-e	37.9	0.5	cd
2BF2	11.0	0.2	k-t	25.5	0.3	o-c′	37.6	0.4	a-d	56.0	0.5	b-e	57.9	0.4	h-l	27.2	0.1	k-s	10.8	0.5	d
2BF3	12.4	0.5	a-j	27.7	0.1	d-g	36.3	0.8	d-g	56.3	0.8	b-c	69.8	0.8	a-b	31.5	0.9	f-j	8.5	1.5	d
2BF4	11.6	0.2	e-s	23.8	0.4	f′-j′	31.6	0.1	q-b′	47.7	0.5	v-d′	56.8	0.3	i-o	29.2	0.4	i-p	17.8	0.8	d
Mean	11.5	0.23	AB	25.8	0.43	B	34.3	0.83	B	53.8	1.1	A	64.2	2.11	A	30.7	0.91	A	18.8		B
AaF-FG (Vertalec×Mycotal)																					
AaF1	12.9	0.0	a-f	26.8	0.5	f-o	32.9	0.3	h-w	48.2	0.1	r-d′	48.0	0.4	v-z	9.1	0.2	u	20.0	0.9	d
AaF2	12.4	0.2	b-k	25.2	0.3	q-f′	34.0	1.0	f-l	46.8	0.2	u-d′	50.7	0.5	w-a′	9.7	0.1	u-v	29.6	1.3	d
AaF3	12.8	0.6	a-f	26.1	0.3	i-x	33.9	0.2	h-q	50.2	0.6	k-x	50.9	0.8	r-w	8.4	0.2	u-v	24.5	4.9	d
AaF6	13.6	0.3	a-b	27.3	0.1	e-k	34.6	0.1	e-j	48.5	0.1	q-d′	49.5	0.2	u-z	9.4	0.2	u-v	26.2	1.3	d
AaF7	11.7	0.2	e-s	24.7	0.1	x-i′	32.9	0.2	h-w	46.7	0.6	z-d′	47.7	0.3	w-a′	9.4	0.1	u-v	30.4	1.4	cd
AaF8	13.9	0.1	a	27.5	0.1	d-i	34.9	0.2	e-h	48.1	0.3	s-d′	50.2	0.2	t-y	9.3	0.3	u-v	26.5	0.4	d
AaF9	13.1	0.6	a-e	26.9	0.0	f-o	33.2	0.3	h-v	48.3	0.1	r-d′	49.0	0.4	u-z	8.2	0.2	v-w	42.6	0.8	cd
AaF11	12.5	0.4	a-i	25.5	0.2	o-d′	33.4	0.3	h-t	48.0	0.3	u-d′	50.0	0.2	u-z	10.0	0.2	u-v	58.9	16	cd
AaF12	12.0	0.2	d-p	26.3	0.2	g-u	34.6	0.2	e-j	49.7	0.4	m-z	47.7	0.1	w-a′	9.1	0.1	u-v	40.1	1.1	cd
AaF17	12.7	0.4	a-h	25.3	0.3	p-e′	33.8	0.2	h-r	48.8	0.4	p-c′	46.8	0.2	y-b′	9.1	0.1	u-v	29.0	2	d
AaF20	12.6	0.4	a-h	26.5	0.1	f-s	33.6	0.0	h-s	47.5	0.2	w-d′	48.0	0.3	v-z	9.1	0.2	u-v	45.9	2.2	cd
AaF24	12.7	0.3	a-h	26.9	0.3	f-o	32.7	0.9	h-x	46.9	0.3	y-d′	48.8	0.4	u-z	8.5	0.2	u-v	29.9	1	d
AaF28	12.7	0.1	a-h	25.6	0.1	n-b′	33.1	0.0	h-v	47.6	0.1	v-d′	48.2	0.1	v-z	8.7	0.1	u-v	49.3	5	cd
AaF30	9.4	0.1	v-w	19.0	0.8	n′	25.1	1.1	e′	60.0	0.8	a	54.2	1.2	m-r	10.2	0.3	u-v	0.9	0.5	d
AaF33	12.5	0.3	a-i	26.7	0.1	f-p	33.2	0.1	h-v	51.2	0.5	i-s	50.6	0.1	s-x	8.7	0.2	u-v	77.1	20	bc
AaF44	11.9	0.7	d-p	26.8	0.1	f-o	32.2	0.2	k-y	49.9	0.5	k-z	49.6	0.7	u-z	9.2	0.1	u-v	18.8	3.8	d
AaF46	13.4	0.4	a-c	27.6	0.2	d-g	34.9	0.0	e-h	52.6	1.4	f-n	54.8	1.7	k-q	9.1	0.1	u-v	13.8	1.2	d
AaF52	12.7	0.0	a-g	26.1	0.2	j-x	33.4	0.2	h-u	52.3	1.4	f-o	49.6	0.3	u-z	8.7	0.2	u-v	28.6	3.5	d
AaF58	9.4	0.2	w	19.7	0.0	m′-n′	25.8	0.0	e′	43.4	0.7	e′-f′	43.0	1.2	c′	10.0	0.2	u-v	62.4	30	cd
AaF69	12.0	0.1	d-p	25.0	0.2	t-g′	33.1	0.2	h-v	47.0	0.2	y-d′	46.7	0.1	z-b′	9.3	0.3	u-v	46.7	7.1	cd

Table 1. (Continued)

Strains	Colony size (diameter: mm)												No. of conidia ($\times 10^6$/cm^2)		
	5°C			10°C			15°C			20°C			25°C			30°C					
	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
AaF-FG (Vertalec×Mycotal)																					
AaF75	11.9	0.1	d-p	24.1	0.2	d′-i′	31.9	0.3	l-z	49.8	0.5	l-z	48.8	0.3	u-z	8.3	0.1	u-v	36.4	16	cd
AaF87	12.1	0.1	d-n	23.8	0.1	e′-j′	33.2	0.2	h-v	48.2	0.5	r-d′	47.7	0.1	w-a′	9.3	0.1	u-v	46.9	9	cd
AaF93	12.2	0.3	b-l	24.9	0.1	t-h′	33.1	0.6	h-v	48.5	0.2	q-d′	50.0	0.4	u-z	8.0	0.0	v-w	33.1	4	cd
AaF99	12.7	0.4	a-h	25.1	0.0	r-g′	32.5	0.1	i-y	53.8	1.6	c-j	50.5	0.2	s-x	8.3	0.4	u-v	24.8	3.4	d
AaF103	12.1	0.1	b-m	26.9	0.1	f-o	34.0	0.2	g-p	51.9	1.2	g-p	47.7	0.6	w-a′	9.2	0.0	u-v	29.5	0.7	d
Mean	12.3	0.13	AB	25.5	0.24	B	32.8	0.27	C	49.4	0.38	B	49.1	0.28	C	9.0	0.07	B	34.9		B
BbF-FG (Vertalec×Mycotal)																					
BbF1	12.1	0.2	b-m	28.6	0.4	c-e	36.8	0.2	c-e	52.4	0.5	f-n	48.9	0.4	u-z	11.1	0.0	u-v	8.0	1.9	d
BbF2	12.4	0.1	a-j	24.7	0.2	w-i′	33.4	0.3	h-v	54.4	0.6	c-i	50.4	0.2	t-x	9.5	0.1	u-v	39.3	1.3	cd
BbF4	11.8	0.1	d-p	27.9	0.7	d-f	36.8	0.6	c-e	52.7	0.4	f-n	47.6	0.2	w-a′	9.5	0.3	u-v	1.9	0.5	d
BbF5	11.0	0.0	k-t	29.0	0.1	b-d	38.7	0.4	a-c	54.9	0.2	c-g	42.6	0.5	c′	8.9	0.1	u-v	107.7	9	bc
BbF6	11.8	0.1	e-q	30.1	0.2	a-b	39.4	0.3	a-b	53.9	0.2	c-j	41.7	0.7	c′	8.7	0.1	u-v	115.6	6.5	ab
BbF10	11.3	0.0	i-t	29.8	0.2	a-c	38.7	0.2	a-c	56.1	0.2	b-d	41.5	0.0	c′	9.8	0.3	u-v	153.6	10	a
BbF12	11.2	0.0	i-t	27.0	0.4	f-n	37.3	0.3	b-d	51.6	0.0	h-q	44.4	0.5	a′-c′	9.2	0.1	u-v	79.1	21	bc
BbF15	11.6	0.2	e-s	30.1	0.1	a-c	37.9	0.2	a-d	53.0	0.1	d-l	43.5	0.8	b′-c′	9.5	0.1	u-v	126.5	16	ab
BbF17	12.0	0.2	d-o	30.9	0.1	a	40.8	0.2	a	58.3	0.5	a-b	41.6	0.2	c′	9.0	0.0	u-v	134.5	35	ab
Mean	11.7	0.09	AB	28.7	0.36	A	37.8	0.4	A	54.2	0.4	A	44.7	0.64	D	9.5	0.14	B	85.1		A
ANOVA α=0.01	$F_{84,170}$=20.19799 p<0.001			$F_{84,170}$=65.2332 p<0.001			$F_{84,170}$=44.8076 p<0.001			$F_{84,170}$=39.79045 p<0.001			$F_{84,170}$=137.1942 p<0.001			$F_{84,170}$=293.3724 p<0.001			$F_{84,170}$=16.21055 p<0.001		

[a] Different capital letters indicate significantly different values (p<0.01) among fusant groups and parental strains according to Scheffe's F-test.

[b] Different small letters indicate significantly different values (p<0.01) among individual tested strains according to Tukey's HSD test. Shaded strains were candidate strains for biological control agent selected by Aiuchi et al. (2007).

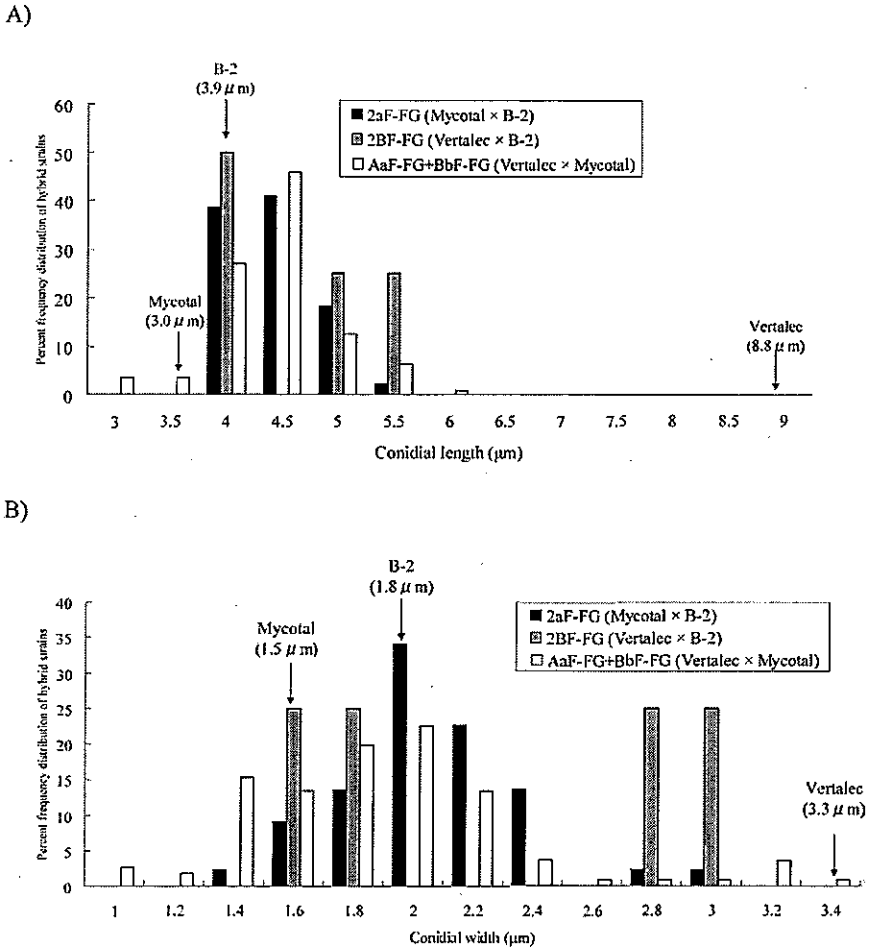

Fig. 2. The percentage frequency distribution of conidial lengths and widths of hybrid strains. (A) conidial lengths and (B) conidial widths of 2aF-FG (n=44), 2BF-FG (n=4), and AaF-FG and BbF-FG (n=111). Arrows show conidial lengths or widths of parental strains.

their growth at low temperatures (10–20°C) (Table 1, Fig. 1); rapid growth of the pathogen under suboptimal temperatures might have a stronger effect on a pest that shows equally good growth at suboptimal temperatures (Hall, 1981). High efficiency of BbF-FG strains at a lower temperature is expected because in addition to high relative humidity during the night under uncontrolled greenhouse conditions, temperatures decrease to 15°C in July and 10–15°C in October in northern Japan. Furthermore, at temperatures between 20 and 30°C, 5 strains of 2aF-FG and 3 strains of 2BF-FG exhibited significantly more rapid colony growth than their parental strains. At 30°C, B-2, 2aF-FG, and 2BF-FG strains showed good growth, while Vertalec®, Mycotal®, AaF-FG, and BbF-FG showed slight growth (Table 1, Fig. 1); the growth characteristics of these strains corresponded to their genotypes (Aiuchi et al., 2008). The colony sizes of AaF-FG and BbF-FG strains incubated at 30°C were less than 10 mm. In contrast, the colony sizes of most 2BF-FG and 2aF-FG strains were more than 25 mm, and the maximum colony size was 41.3 mm (Table 1).

Conidial production was highly diverse and showed succession among the tested strains ($F_{84,170}$=16.21055; p<0.001) (Table 1). For example, BbF10 showed the highest conidial production (153.6×10^6 conidia/cm^2), while the AaF30 strain showed the lowest production (8.9×10^5 conidia/cm^2). There was a significant difference of about 170 times in conidia production between AaF30 and BbF10 strains, although both hybrid strains showed Mycotal®-type profiles in genomic DNA

analysis by AP-PCR and were derived from Vertalec® and Mycotal® as parents (Aiuchi et al., 2008). Although BbF-FG strains showed a tendency toward higher conidia productivity than the other tested strains, the majority of 2aF-FG and 2BF-FG strains showed a tendency to produce fewer conidia than their parents. Alteration in the growth rate and sporulation of intrastrain fusants of *Trichoderma reesei* have been observed, and all fusant strains were reported to show luxuriant growth and profuse sporulation (Prabavathy et al., 2006). Drummond and Heale (1988) reported various amounts of spore production in recombinant strains of *V. lecanii* generated by hyphal anastomosis. They concluded that the change in sporulation ability was caused by mitotic crossing over or chromosome reassortment events. It can be presumed that the varied conidiation ability of hybrid strains of *V. lecanii* was also a result of recombination events triggered by protoplast fusion.

Figure 2 shows the distribution frequency of the conidial length and width of the tested strains. The conidial length of the tested strains (except Vertalec®; 8.8±0.3 μm) ranged from 2.5±0.1 (AaF36) to 5.6±0.1 μm (AaF53); thus, it showed continuous variation. The conidial width of the tested strains also showed continuous variation and ranged from 0.9±0.03 (AaF36) to 3.3±0.1 μm (Vertalec®). With regard to conidial length, Vertalec® exhibited significantly longer conidia than all other tested strains. Among 2aF-FG and 2BF-FG strains (both FGs showing B-2 type profiles in genomic DNA analysis by AP-PCR; Aiuchi et al., 2008), the conidial lengths of 7 (15.9%) and 1 (25.0%) strains, respectively, were significantly greater than that of B-2; furthermore, among BbF-FG and AaF-FG strains (both FGs showing Mycotal® type profiles in genomic DNA analysis by AP-PCR; Aiuchi et al., 2008), the conidial lengths of 15 and 81 strains (86.5%), respectively, were significantly greater than that of Mycotal®. With regard to conidial width, Vertalec® showed significantly wider conidia than all other tested strains (except for 5 BbF strains). The conidial widths of 16 (36.4%) and 2 (50.0%) strains of 2aF-FG and 2BF-FG, respectively, were significantly greater than that of B-2, and the conidial widths of 14 and 41 (49.5%) strains of BbF-FG and AaF-FG, respectively, were significantly greater than that of Mycotal®. Jackson and Heale (1987) reported a varying relationship between the DNA content of the conidium and conidial volume, but they suggested that conidial size was not a reliable indicator of ploidy in the intraspecific hybridization of *V. lecanii* via hyphal anastomosis or protoplast fusion; however, Lalithakumari (2000) reported that the spore sizes of fusants of *T. harzianum* and *T. longibrachiatum* were intermediate to those of both parental strains, along with a slight increase in the DNA content of fusants per conidium. In our study, hybrid strains showed a tendency toward slight conidia enlargement as compared to their parents of the same genotype. The study of the DNA content of the conidium remains a subject of our future research.

In addition to genetic diversity, colony morphology (Aiuchi et al., 2008), virulence against insect pests, and viability on the leaf surface (Aiuchi et al., 2007), the radial growth rate, conidial production, and conidial size varied in individual hybrid strains of *V. lecanii*. Hall (1984) found that *V. lecanii* strains with large spores and a high germination rate showed strong epizootic potential against *Macrosiphoniella sanborni*. According to a study by Jackson et al. (1985), the high germination rate, high sporulation, absence of amylase activity, and high chitinase production were frequently associated with the virulence of *V. lecanii* against *M. sanborni*. In contrast, Drummond et al. (1987) reported no correlation between the conidiospore size or germination rate of *V. lecanii* and virulence against greenhouse whitefly. In this study, no apparent relationship was found between the colony growth rate, conidial production ability, or conidial size and mortality of cotton aphid or greenhouse whitefly (Aiuchi et al., 2007). Earlier studies on the correlation between virulence and other fungal traits concluded that several different traits are associated with virulence or epizootics (Hall, 1984; Jackson et al., 1985; Drummond et al., 1987). Our data also indicated varying degrees of relationship between virulence to insect pests and fungal traits.

Of the 13 hybrid strains selected as candidates for mycoinsecticides in our previous study (Aiuchi et al., 2007), 11 belonged to 2aF-FG or 2BF-FG; both these FGs were able to grow at 30°C and showed faster growth at 25°C (Table 1). Furthermore, highly virulent strains of 2aF4 and 2aF43 among 2aF-FG and 2BF-FG strains showed higher

conidia productivity on solid medium (Table 1); 2aF43 also showed high viability on the leaf surface under low humidity conditions. Profuse conidia production represents not only high virulence but also important traits to achieve high productivity when the formulation is prepared. A further study of conidial traits of hybrid strains, including the study of the germination rate, conidia productivity on insect cadavers, and mode of conidiation, should be conducted. Yeo et al. (2003) suggested that the screening procedure should follow a "biorational approach" based on the selection of isolates on many traits, including the ability to grow and germinate quickly at temperature and humidity conditions under which the mycoinsecticide would be used, virulence to the target pest, production potential, and compatibility with chemical pesticides and other natural enemies. This method enables the selection of more appropriate isolates that possess the ability to operate over a range of abiotic conditions under which they will be used, with little impact on nontarget natural enemies. Subsequently, biorational selection for epiphytic ability will be carried out to select the most appropriate hybrid strain from among the candidates for greenhouse use. This strain can be incorporated into an integrated pest management program from the perspective of preventive biological control.

Apparently, the alteration in fungal traits that occurred during the sequential process of protoplast fusion engendered the variety of hybrid strains described herein. Thus, protoplast fusion techniques can be used to generate hybrid strains that have various intrinsic fungal properties.

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