Independence of Sexual and Vegetative Incompatibility Mechanisms of *Thanatephorus cucumeris* (*Rhizoctonia solani*) Anastomosis Group 1

María C. Julián, Fons Debets, and Jaap Keijer

First and third authors: Department of Virulence and Resistance, DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, Netherlands; second author: Department of Genetics, Agricultural University of Wageningen, Wageningen, Netherlands; third author: Department of Microbiology and Biotechnology, Rikilt-DLO, Postbus 230, 6700 AE, Wageningen, Netherlands.

M. C. Julián was supported by a grant from the European Union (AIR3-BM93-1107).

We thank D. Carling for providing us with the parent isolate used in this study. We also thank P. van den Boogert, A. Dullemans, R. Jansen, C. van Silfhout, and C. Waalwijk for critically reviewing this manuscript.

Accepted for publication 22 February 1996.

ABSTRACT

Julián, M. C., Debets, F., and Keijer, J. 1996. Independence of sexual and vegetative incompatibility mechanisms of *Thanatephorus cucumeris* (*Rhizoctonia solani*) anastomosis group 1. Phytopathology 86:566-574.

The soilborne plant pathogen *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*) is a basidiomycete that occurs worldwide and causes damage to a large variety of agricultural crops. The lack of knowledge of the genetic basis of incompatibility in *T. cucumeris* hampers the development of environmentally friendly control measures for this plant pathogen. To clarify incompatibility mechanisms in *T. cucumeris*, sexual and vegetative compatibility were investigated simultaneously in anastomosis group (AG)-1. Sporulation was induced in vitro for a field isolate belonging to AG-1, and single spores were isolated, giving rise to homokaryotic colonies. The homokaryons were paired, and the contact area between isolates was studied macro- and microscopically. Mating processes (formation of heterokaryotic tufts between paired homokaryons) occurred independently from vegetative incompatibility processes (lysis of anastomosed cells), showing that in *T. cucumeris* AG-1 sexual and vegetative incompatibility are two mechanisms that operate independently. Vegetative incompatibility was variable and irreproducible, indicating vegetative compatibility in *T. cucumeris* AG-1 is a complex mechanism. Furthermore, heterokaryotization of homokaryotic mycelium (Buller phenomenon) was observed. A novel phenomenon is described, consisting of the spontaneous lysis of the cells of some of the homokaryotic progeny of the field isolate.

Rhizoctonia solani Kühn is the asexual form of the fungal species *Thanatephorus cucumeris* (Frank) Donk (1,3,30). *R. solani* is cosmopolitan in soils and is a destructive plant pathogen with a wide host range (1). The large variation between isolates of *R. solani* with respect to pathogenicity and growth characteristics and the concurrent lack of knowledge of the genetic basis of this variation make it very difficult to understand the population structure of this fungus. Insight into the genetics of *R. solani* is necessary to understand how the flow of genetic information within and between populations takes place. This knowledge will aid our understanding of the strategies for spread and survival of *R. solani*, facilitating the development of effective environmentally friendly control measures for this plant pathogenic fungus.

Insight into the occurrence of host plant resistance to the various diseases caused by *R. solani* is difficult to obtain because the fungus is not a homogeneous species but, instead, is composed of at least 12 groups (10,34). Isolates of *R. solani* are assigned to anastomosis groups (AGs) based on the occurrence of hyphal fusion (anastomosis) with members of designated AGs (34); isolates from the same AG anastomose, whereas, in general, isolates from different AGs do not anastomose. There are some exceptions, for example AG-BI isolates, the "bridging" isolates, are able to anastomose not only with members of AG-BI, but also with isolates belonging to AGs-2, -3, -6, and -8 (34). When two isolates from the same AG are paired and subsequently anastomose, there are two possible reactions: perfect fusion (a C3 reaction) or imperfect fusion (a C2 reaction) (9). Perfect fusion is the complete fusion of cell walls, membranes, and cytoplasms with continuous living cytoplasm in the fusion site; perfect fusion occurs when anastomosed isolates are vegetatively compatible. Imperfect fusion results in lysis of anastomosing and adjacent cells because anastomosed isolates are vegetatively incompatible.

Studies on the genetics of *T. cucumeris* (*R. solani*) have been carried out in many of the AGs by studying the formation of heterokaryotic tufts arising in the area of contact between single-spore homokaryotic cultures (27,31,35). Until now, only the genetics of AGs-1, -4, and -8 were understood to a certain extent (1, 31,36). Little is known about the sexual compatibility of the remainder of the AGs (1), but it has been suggested that the genetics of AGs-2 and -3 differ from that of AGs-1 and -4 (1). In AGs-1 and -4, heterokaryon formation between paired homokaryons is controlled by two closely linked genes, which together are referred to as the H factor (5). Paired homokaryons that carry H factors differing at one or both genes produce fluffy tufts of heterokaryotic hyphae. The H factor in *T. cucumeris* has been described as controlling heterokaryon formation (5) by functioning like the incompatibility loci in other basidiomycetes. In later studies, it was recognized that the H factor also controls nuclear pairing and the stability and outbreeding of the heterokaryon (31).

Based on these observations, Adams and Butler (2) have suggested that heterokaryon incompatibility factors (H factors) in *T. cucumeris* are the same as sexual incompatibility factors. Also, Anderson (4) stated that sexual and vegetative incompatibility systems function simultaneously and reciprocally. To consider sexual and vegetative incompatibility as one and the same mechanism in *T. cucumeris* would contradict what is found in most species of higher fungi. In many species of ascomycetes and basidiomycetes,

Corresponding author: J. Keijer; E-mail address: j.keijer@rikilt.dlo.nl

Publication no. P-1996-0326-05R
© 1996 The American Phytopathological Society

the existence of two distinct types of incompatibility, sexual and vegetative, that serve contrasting but complementary roles (32) has been described.

To clarify incompatibility in *T. cucumeris*, sexual and vegetative compatibility in AG-1 were studied simultaneously. Vegetative compatibility was assessed microscopically as perfect fusion, with no cell lysis after anastomosis (4). Sexual compatibility was assessed macroscopically as formation of heterokaryotic tufts when homokaryons of different mating types were paired (5,18,35). The fact that the tufts arising at the junction between paired homokaryons belonging to AGs-1 and -4 are heterokaryotic has been demonstrated by a number of researchers using naturally occurring markers (5,35), mutants (27,31), sporulation behavior (2), virulence (18), and randomly amplified polymorphic DNA markers (13).

If in *T. cucumeris* sexual and vegetative compatibility were the same mechanism, then formation of a heterokaryon when pairing homokaryons with different mating types (sexual compatibility) should be accompanied by perfect fusion (vegetative compatibility). Likewise, the lack of formation of a heterokaryon when pairing homokaryons with the same mating type (mating incompatibility) should result in cell lysis of the anastomosed cells (vegetative incompatibility).

In this report, we present our studies of pairings between sibling single-spore isolates (SSIs) belonging to AG-1, and evidence is given for independence between sexual and vegetative incompatibility systems in *T. cucumeris* AG-1. In addition, based on the fact that small tufts occasionally form when a heterokaryon is paired with a homokaryon, we suggest that heterokaryotization of a homokaryon by a heterokaryon (Buller phenomenon) might take place in *T. cucumeris*. Furthermore, spontaneous lysis of hyphae was observed in some of the homokaryotic isolates, a phenomenon that has not been reported before, to our knowledge, in *T. cucumeris*.

MATERIALS AND METHODS

Isolates and internal transcribed spacer-polymerase chain reaction (ITS-PCR) subgroup determination. The heterokaryotic *T. cucumeris* (*R. solani*) field isolate 1R4 used in this study was provided by D. E. Carling (University of Alaska Fairbanks) and corresponds to number 43 in his collection. The AG of this isolate was confirmed by pairing it with AG tester isolates (34) as described by Keijer et al. (20). SSIs derived from single 1R4 basidiospores are denoted as *1 through *46. Isolate *4 was lost during this study. AG-subgroup determination was confirmed by amplification of the ITS of the ribosomal gene cluster by PCR followed by digestion of the products with the restriction endonuclease *Eco*RI (20). This was done for the parent isolate 1R4 as well as for the derived SSIs *2, *3, *12, and *15; 10 ng of fungal DNA from the parent and the SSIs was used as template in ITS-PCR, which was performed as described by Keijer et al. (20) with the primers ITS1: 5′-TCCGTAGGTGAACCTGCGG and ITS4: 5′-TCCTCCGCTTATTGATATGC. The products were digested with endonuclease *Eco*RI (Life Technologies, Bethesda, MD) at 37°C for 2 h, separated by electrophoresis in 1% agarose (33), stained with ethidium bromide, and photographed over a UV transilluminator. Total DNA extracted from field isolate 1R4 and SSIs *2, *3, *12, and *15 was visualized on agarose gels (33) to determine whether plasmids were present. These DNAs also were separated by cesium chloride gradients (33) to determine the presence of a plasmid band.

Media. Potato marmite dextrose agar (PMDA) contained 39 g of potato-dextrose agar (PDA, Oxoid, London) and 1 g of marmite yeast and vegetable extract (CPC Ltd., Esher, Surrey, England) per liter. Water agar (WA) was 15 g of agar technical no. 3 (Oxoid) per liter. Potato-dextrose charcoal agar (PDCA) contained 39 g of PDA supplemented with 0.5% (wt/vol) activated charcoal (Sigma Chemical Company, St. Louis) per liter. WA covered with cellophane (WA + cellophane) was prepared by autoclaving cut pieces of cellophane and placing them on top of a WA plate. Fortified nutrient agar (FNA) contained 20 g of agar technical no. 3, 15 g of sucrose, 2 g of asparagine, 0.6 g of KH_2PO_4, 0.8 g of K_2HPO_4, 1 g of $MgSO_4$, 10 mg of $CaCl_2$, 2.5 mg of $ZnSO_4 \cdot 7H_2O$, 2.5 mg of H_3BO_4, 0.5 mg of $MnSO_4 \cdot 1H_2O$, 1.7 mg of NaFeEDTA, 0.3 mg of $CuSO_4 \cdot 5H_2O$, and 0.1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, as well as 1.0 mg of thiamin, 1.0 mg of niacin, 20 µg of biotin, 0.5 mg of Ca-pantothenate, 0.5 mg of pyridoxine, and 0.1 mg of *p*-aminobenzoic acid per liter. All reagents were analytical grade and were obtained from Sigma or Merck (Darmstadt, Germany).

Sporulation and isolation of SSIs. Isolate 1R4 was induced to fruit as follows. A petri dish with PMDA was inoculated with a 3-mm^3 agar plug from the stock culture and incubated for 30 days at 23°C in the dark. A small agar plug was cut from the edge of the colony and placed in a 9-cm-diameter petri dish with WA. The WA plates were incubated at 23°C in the dark until the mycelium covered the plate completely and then were placed on a laboratory bench in diffuse light. In fruiting experiments, petri dishes with supports in the lid were used to facilitate aeration. Of the AG-1 isolates tested for fruiting, only isolate 1R4 fruited reproducibly and abundantly.

SSIs were isolated by inverting a sporulating culture overnight over WA plates. Germinated single basidiospores were isolated 1 to 6 days later by picking them from the agar with a sterile Pasteur pipette under an inversion microscope and transferring them to WA plates. The single spores were incubated at 23°C in the dark. When mycelial colonies began to develop, hyphal tips were transferred to FNA plates. The SSIs as well as the parent isolate were stored at 4°C on FNA.

Pairings of SSIs and formation of heterokaryons. Pairings among the SSIs were performed on PDCA. This medium has been widely applied in analysis of the genetics of *T. cucumeris* (7,8). The disadvantage of this nontranslucent growth substrate is that it does not allow microscopic observation of anastomosis. Therefore, WA + cellophane also was employed. Plugs (3-mm^3) of each isolate were placed 6 cm apart in a 9-cm-diameter plate and incubated at 23°C in the dark. Observations of the plates were done during a period of 14 days. Tuft formation (sexual compatibility) was studied macroscopically on both media. Anastomosis was studied microscopically on WA + cellophane. Perfect anastomosis or C3 reaction (vegetative compatibility) was reported when anastomosed and adjacent cells were not lysed and the diameter of anastomosis points was equal to the hyphal diameter. Imperfect anastomosis or C2 reaction was reported when anastomosed and adjacent cells lysed and the diameter of anastomosis points was less than the hyphal diameter. When tufts of aerial mycelium formed in the junction between paired SSIs, hyphae from each tuft were picked with sterile forceps and placed on WA. When colonies began forming, hyphal tips were transferred to FNA. To confirm that the picked hyphae from the junction were formed heterokaryons, the test proposed by Adams and Butler (2) was performed. Cultures derived from the tufts were paired to both parent homokaryons in PDCA plates. Lack of tuft formation with both of the homokaryotic parents was considered adequate proof that the heterokaryon had been successfully formed and isolated.

RESULTS

Sporulation and subgroup determination. *T. cucumeris* (*R. solani*) field isolate 1R4 sporulated easily in culture, and the spores germinated readily on WA. The SSIs obtained from 1R4 displayed a large variation in cultural characteristics such as color, sclerotia formation, and growth rate. ITS-PCR subgroup determination confirmed that parent isolate 1R4 as well as the derived SSIs belong to AG-1-IC, the microsclerotial form of AG-1 (Fig. 1).

Pairings of SSIs. The pairing medium WA + cellophane was a highly suitable medium for analyzing compatibility reactions, be-

cause the cellophane prevented *T. cucumeris* from growing in the agar, allowing for observation of the cellular interactions in one mycelial layer. In addition, the cellophane reduced contamination of the plates during microscopic observations, because it apparently does not provide a suitable growth surface for most microorganisms. Furthermore, observations during several weeks were not hindered by cell death. If WA alone is used, hyphae on the surface die after a few days. On WA + cellophane cell death did not occur, enabling study of the reactions that took place when homokaryons were paired over a period of 14 days.

Preliminary studies were done for which randomly chosen SSIs *2 and *5 were paired with SSIs *1 and *3 and with themselves on WA + cellophane. The interactions in the area of contact between the isolates were studied. No tuft formation was observed in any of the pairings. Most pairings showed perfect anastomosis or vegetatively compatible interaction: no lysis could be observed from the moment of first contact between the isolates up to 8 days later. Only two combinations, *2 × *3 and *5 × *3 showed imperfect anastomosis or vegetatively incompatible interaction: cell lysis was observed from the first day of isolate contact. We concluded that there appeared to be different vegetative compatibility groups of SSIs. One group was represented by SSIs *1, *2, and *5, and the other group was represented by SSI *3. As expected, self pairings resulted in a compatible reaction.

For further experiments, one SSI from each vegetative compatibility group was randomly chosen (SSIs *2 and *3, respectively) and paired to all SSIs, both on WA + cellophane and PDCA. The contact area was studied at 6, 10, and 14 days after inoculation for vegetative and sexual compatibility. Each pairing was duplicated, and the experiment was repeated three times. The results are shown in Table 1. In addition, parent isolate 1R4 was paired with all the SSIs and with itself.

These experiments allowed us to make some observations. First, tuft formation did not depend on the occurrence of perfect anastomosis (vegetative compatibility), just as the lack of tuft formation did not imply imperfect anastomosis (vegetative incompatibility). Tuft formation occurred clearly in the presence and absence of cell lysis, just as absence of tuft formation took place with and without cell lysis. For example, SSIs *6 and *22 formed a tuft with SSIs *2 and *3; however, SSI *6 showed no cell lysis with SSIs *2 or *3, whereas SSI *22 showed lysis with both SSIs *2 and *3. Likewise, SSIs *5 and *20 did not form a tuft when paired with SSIs *2 or *3; however, SSI *5 showed no cell lysis when paired with SSIs *2 and *3, and SSI *20 showed cell lysis when paired with SSIs *2 and *3 (these examples are italicized and underlined in Table 1). The independence of tuft formation (sexual compatibility) and vegetative compatibility is illustrated in Figure 2, in which tuft formation and a barrage reaction occurred simultaneously all along the area of contact. The barrage corresponded microscopically with cell lysis in the area of anastomosis.

Second observation: the formation or nonformation of a tuft was consistently reproducible between experiments, whereas cell lysis after anastomosis was less reproducible (Table 1). Variation in compatibility responses was observed even within one pairing, where along the contact area between two paired isolates some

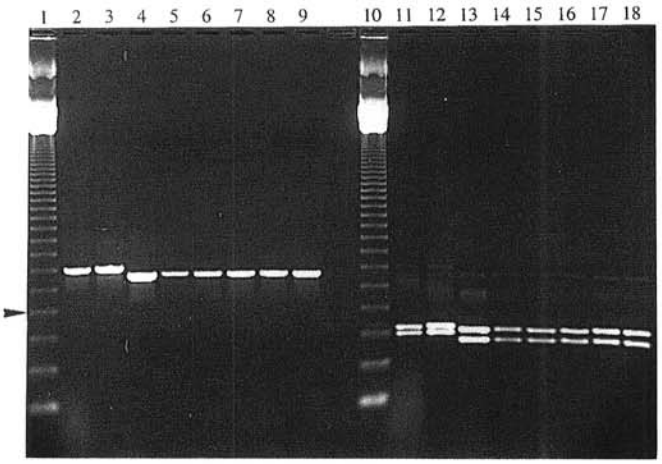

Fig. 1. Internal transcribed spacer-polymerase chain reaction (ITS-PCR) of *Thanatephorus cucumeris* parent isolate 1R4 and single-spore isolates (SSIs) *2, *3, *12, and *15. Lanes 1 and 10, 123-bp ladder. The 429-bp band is indicated with an arrow. Lanes 2 to 9, full-length ITS-PCR products. Lanes 11 to 18, ITS-PCR products digested with *Eco*RI. Lanes 2 and 11, tester anastomosis group (AG)-1-IA. Lanes 3 and 12, tester AG-1-IB. Lanes 4 and 13, tester AG-1-IC. Lanes 5 and 14, parent isolate 1R4. Lanes 6 and 15, SSI *2. Lanes 7 and 16, SSI *3. Lanes 8 and 17, SSI *12. Lanes 9 and 18, SSI *15.

TABLE 1. Sexual and vegetative compatibility of single-spore isolates (SSIs) *2 and *3 of *Thanatephorus cucumeris* and vegetative compatibility of parent isolate 1R4 with progeny SSIs at 10 days after inoculation[y]

	Sexual compatibility		Vegetative compatibility		
SSIs	*2	*3	*2	*3	1R4
*1	I	I	C	i/i/c	C
*2	I	I	i/c/c	I	C
*3	I	I	I	C	i/c/i
*5	*I*	*I*	*C*	*C*[z]	C
*6	*C*	*C*	*C*	*C*	C
*7	C	C	c/c/i	I	i/c/i
*8	C	C	c/i/c	i/c/c	C
*9	C	C	c/i/i	i/i/c	c/c/i
*10	I	I	c/i/i	I	C
*11	C	C	C	i/c/c	C
*12	C	C	i/c/i	I	I
*13	I	I	i/i/c	i/c/i	i/i/c
*14	I	I	C	I	I
*15	C	C	c/c/i	I	C
*16	C	C	i/c/i	I	i/c/i
*17	C	C	c/c/i	I	I
*18	I	I	I	c/i/c	C
*19	I	I	i/c/i	I	C
*20	*I*	*I*	*I*	*I*	I
*21	C	C	i/i/c	I	C
*22	*C*	*C*	*I*	*I*	I
*23	I	I	C	I	C
*24	C	C	i/c/i	i/c/i	c/i/i
*25	I	I	I	I	C
*26	I	I	C	c/i/i	C
*27	I	I	i/c/i	c/c/i	I
*28	C	C	c/i/i	c/i/i	C
*29	C	C	i/c/i	I	C
*30	I	I	c/i/c	c/i/c	C
*31	C	C	I	I	i/c/i
*32	C	C	c/i/i	I	C
*33	C	C	C	I	C
*34	C	C	i/c/i	I	I
*35	C	C	c/i/i	i/c/i	i/c/c
*36	C	C	C	I	C
*37	I	I	c/c/i	I	c/i/c
*38	C	C	I	I	i/i/c
*39	I	I	C	i/c/i	C
*40	I	I	I	c/i/i	I
*41	I	I	C	c/i/c	c/i/c
*42	I	I	i/i/c	I	C
*43	I	I	c/c/i	I	c/i/i
*44	C	C	I	I	I
*45	C	C	i/c/i	i/c/i	C
*46	C	C	c/i/i	I	C

[y] In sexual compatibility: C = sexual compatible (tuft); I = sexually incompatible (no tuft). In vegetative compatibility: C = vegetatively compatible (no lysis when anastomosed); I = vegetatively incompatible (lysis when anastomosed). Where vegetative compatibility was not reproducible between experiments, the results of the three experiments are shown in lowercase letters. Examples given in the text are italicized and underlined and show independence of sexual and vegetative incompatibility mechanisms.

[z] In additional pairing experiments, including the preliminary experiment, an incompatible reaction (C2 reaction) was sometimes observed in anastomosis between these isolates.

areas showed lysis (vegetative incompatibility) and some did not. Furthermore, even in self pairing, incompatible reactions were observed in one of the experiments (Table 1, SSI *2). In addition, a large variation in the intensity of the killing reaction was observed. In some pairings, only the anastomosed cells lysed, whereas in other pairings lysis affected not only the anastomosed cells but also many of the neighboring cells. In some cases, lysis was so extended that it could be observed macroscopically as a barrage (Fig. 2). In pairings of the parent 1R4 with its homokaryotic progeny, cell lysis after anastomosis was observed clearly in 9 of the cases; in 24 of the cases, no cell lysis was observed; and the remaining 12 pairings gave no clear reaction (Table 1).

Third observation: based on the lack of tuft formation, we concluded that SSIs *2 and *3, which belong to different vegetative compatibility groups, belong to the same sexual compatibility group or mating type. Based on the results of the sexual compatibility reactions, the SSIs could be divided into two sexual compatibility (mating type) groups of almost equal size. The sexual compatibility types were arbitrarily named M1 and M2. M1: SSIs *1 to *3, *5, *10, *13, *14, *18 to *20, *23, *25 to *27, *30, *37, *39, and *40 to *43; M2: SSIs *6 to *9, *11, *12, *15 to *17, *21, *22, *24, *28, *29, *31 to *36, *38, and *44 to *46. To confirm the grouping of SSIs according to their mating type, randomly chosen SSIs *2, *3, *12, and *15 were paired to the rest of the SSIs on PDCA and WA + cellophane, and tuft formation was assessed. All pairings were done in triplicate. The results confirmed the mating-type grouping of SSIs.

Fourth observation: in some of the pairings of parent isolate 1R4 with its progeny, very small and discontinuous tufts were observed in the contact area (Fig. 3). In most of the pairings, hyphae from parent and SSI came in contact and showed no tuft formation, whereas in pairings of the parent with SSIs *2, *15, *17, *19, *32, *36, *38, and *41, very small spots of thin aerial hyphae were observed. These spots were fluffy but not as big or spectacular as the tufts formed between pairs of single spore-derived homokaryons. Also, in pairings between in vitro-generated heterokaryons with their parent homokaryons, small spots of fluffy tufts were sometimes observed (Fig. 3).

Fifth observation: large areas of dead cells were observed in some pairings after 2 weeks of incubation. When inoculated, both isolates of the pairing grew toward each other, covering the plate, and formed a tuft if they were of different mating types, after which some cells lysed spontaneously. The lysis process continued until most mycelial cells were dead. Two weeks after inoculation, living hyphae were observed only in the formed tufts and very close (5 to 10 mm) to the inoculum. This process did not take place in pairings between all isolates. To our knowledge, spontaneous lysis has not been reported in *T. cucumeris*.

To exclude the possibility of transfer of "deleterious" material from one isolate to another, thereby generating lysis, each SSI was inoculated separately on WA + cellophane and PDCA plates and studied for 4 weeks. On PDCA, the spontaneously lysing isolates grew very sparsely without formation of sclerotia, whereas the nonlysing isolates grew very densely and formed sclerotia (Fig. 4). On WA + cellophane, all isolates grew, covering the whole surface of the plate, but after a number of days, several isolates began to show lysis of cells in some areas. These areas gradually increased in size until all hyphae were dead, except those close to the inoculum (Fig. 5). Observations through a microscope showed that the lysis always began in lateral branches of the hyphae and then spread through the hyphae. Two weeks after inoculation, 18 of the SSIs showed cell lysis over the entire plate, and no sclerotia were formed; these were described as "spontaneously lysing" isolates. Twenty-six of the SSIs showed no cell lysis, even after 4 weeks, and formed sclerotia; these were described as "nonlysing" isolates. One of the SSI, *23, showed both cell lysis and very weak formation of sclerotia. Grouping of the SSIs regarding lysis and mating type is shown in Table 2. Parent isolate 1R4 showed no lysis and abundant formation of sclerotia. On FNA and PMDA, all isolates grew very densely, forming sclerotia, and no lysis was observed even months after inoculation.

Finally, both on PDCA and WA + cellophane, two types of tufts with different morphology were observed (Fig. 6). One type was continuous along the entire contact area between the paired homokaryons and consisted of long, dry threads of mycelium. Usually, sclerotia were formed on the tuft some time after its formation. This tuft was designated "fibrous tuft." The other type, designated "compact tuft," appeared as discrete spots of aerial mycelium, in some cases as only one spot in the entire contact area between the isolates. The compact tuft contained many tiny drops of water in the branched mycelia, and sclerotia never formed. Fibrous tufts were formed in pairings between nonlysing isolates or between a nonlysing and a spontaneously lysing isolate. Compact tufts were formed only when two spontaneously lysing isolates were paired. Compact tufts remained restricted to the area of contact between homokaryons. The same was true for fibrous tufts formed between two nonlysing isolates, but when a spontaneously lysing isolate was paired with a nonlysing isolate, the fibrous tuft overgrew the spontaneously lysing isolate, in some cases covering the entire surface previously colonized by the spontaneously lysing isolate (Fig. 7). Both types of tuft were visible as abundant aerial growth and were very different from tufts observed between homokaryotic isolates of AG-2 and -3 (M. C. Julián, and J. Keijer, *unpublished data*), between heterokaryotic isolates of AG-3 (M. C. Julián, and J. Keijer, *unpublished data*), and in pairings of AG-8 isolates (36), which have a feltlike appearance rather than the fluffy appearance of the sexual tufts.

Formation of heterokaryons. Heterokaryons were formed by pairing nine homokaryons (underlined in Table 2) on PDCA in all of the possible combinations that were allowed for the mating types and subsequently isolating mycelium from the tuft that was formed in the area of contact between the homokaryons. To test whether heterokaryons had been formed successfully, they were paired on a PDCA plate with both parent homokaryons. In all cases, the control pairings between the parent homokaryons showed very clear and spectacular tuft formation. Most of the newly formed heterokaryons did not produce tufts when paired with their parent homokaryons. However, in 5 of 20 pairings, very small spots of fluffy tuft appeared. These tufts had the same appearance as the ones described in pairings of the parent 1R4 with its progeny.

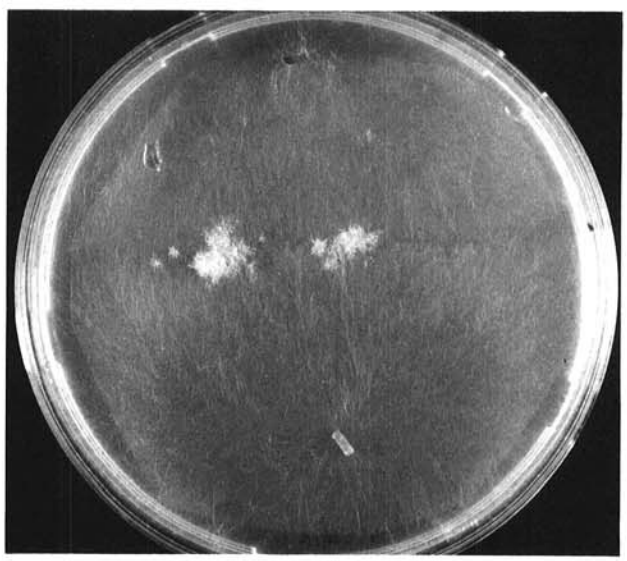

Fig. 2. Pairing between *Thanatephorus cucumeris* single-spore isolates *3 and *44 on water agar plus cellophane shows formation of a compact tuft and, at the same time, a barrage.

The formed heterokaryotic tufts also were tested for spontaneous lysis by inoculating them on WA + cellophane and PDCA. The heterokaryons originating from the pairings of two homokaryotic spontaneously lysing isolates showed spontaneous lysis and no formation of sclerotia. When one or both of the paired homokaryons was a nonlysing isolate, the resulting heterokaryon showed no spontaneous lysis.

DISCUSSION

ITS-PCR subgroup determination. The SSIs obtained from *T. cucumeris* (*R. solani*) AG-1 field isolate 1R4 displayed a large variation in cultural characteristics, indicating the heterokaryotic nature of the field isolate (7,16,18). It is most likely that the variation is due to segregation and recombination of chromosomes during meiosis (20). However, meiotic recombination does not seem to affect the ITS region, because subgroup determination of field isolate 1R4 and the SSIs gave identical results and confirmed that they belong to AG-1-IC, the AG-1 microsclerotial type. In AG-1, only AG-1-IC isolates have been described to fruit in vitro, whereas AG-1-IA and -1-IB have not (3). The subgroups within AG-1 can be distinguished on the basis of pathogenicity (30), cultural characteristics (29), and DNA base sequence homology (22). These methods have the disadvantage of not being very discriminating or requiring substantial effort. ITS-PCR provides a highly discriminating alternative for identifying subgroups of AG-1, both for heterokaryotic as well as for homokaryotic isolates.

Sexual and vegetative incompatibility mechanisms. The acceptance of sexual and vegetative incompatibility as one mechanism in *T. cucumeris* differs substantially from the situation found in other higher fungi, in which vegetative and sexual incompatibility are controlled by distinct incompatibility systems (19). In the ascomycetes *Neurospora crassa*, *Cochliobolus heterostrophus*, and *Podospora anserina*, insight into the genetic basis of incompatibility has been obtained through isolation of mating-type (12, 19) and vegetative incompatibility genes (6). For these fungi, vegetative incompatibility genes do not interfere with sexual reproduction (6), although in *N. crassa* mating-type genes have a dual function (6). In the basidiomycete *Coprinus cinereus*, mating-type genes are nuclear encoded (26), whereas vegetative incompatibility is controlled by the mitochondrial genome (25). Our research proves that in *T. cucumeris*, as in other higher fungi, mating type and vegetative incompatibility are two distinct mechanisms that operate independently.

The terminology employed to define incompatibility in *T. cucumeris* is confusing. For example, Anderson et al. (5) used the term "heterokaryon incompatibility system" to describe vegetative incompatibility in pairings between heterokaryotic field isolates of AGs-1 and -4. The term heterokaryon incompatibility is widely used to describe vegetative incompatibility in homothallic ascomycetes but is confusing in *T. cucumeris*, because vegetative incompatibility is expressed not only in pairings between heterokaryons but also in pairings between homokaryons. In addition, heterokaryon incompatibility could be interpreted as the incompatibility between homokaryons to form a heterokaryon and, thus, refers to sexual incompatibility not vegetative incompatibility. Other confusing terminology involves terms defining groups of isolates capable of hyphal fusion. In most higher fungi, the term vegetative compatibility group is used. In *T. cucumeris*, AG is used for groups of isolates capable of hyphal fusion. Within an AG, isolates may show perfect (vegetative compatibility) or imperfect anastomosis (vegetative incompatibility). Finally, the use of heterogenic incompatibility to describe vegetative and homogenic incompatibility, which describe sexual incompatibility (4), seems premature because no information is available on the genes involved in both types of incompatibility.

The use of a few clearly defined terms to describe interactions in *T. cucumeris* would greatly benefit characterization of *T. cucumeris* with respect to genetics and population structure. We propose the term "sexual" or "mating-type" compatibility when paired homokaryons from the same AG are able to form a distinct heterokaryon and the term "vegetative" or "somatic" compatibility when pairings within an AG between homokaryons, field isolates (heterokaryons), or homokaryon and heterokaryon result in fully vegetatively compatible reactions or perfect anastomosis (C3 reaction, no lysis of anastomosed cells). Vegetative or somatic incompatibility should be used when pairings within an AG between homokaryons, heterokaryons, or homokaryon and heterokaryon result in a vegetative incompatible reaction or imperfect anastomosis (C2 reaction, lysis of anastomosed cells when anastomosed).

Vegetative incompatibility and heterokaryon formation. Studies of vegetative incompatibility in ascomycetes have shown that

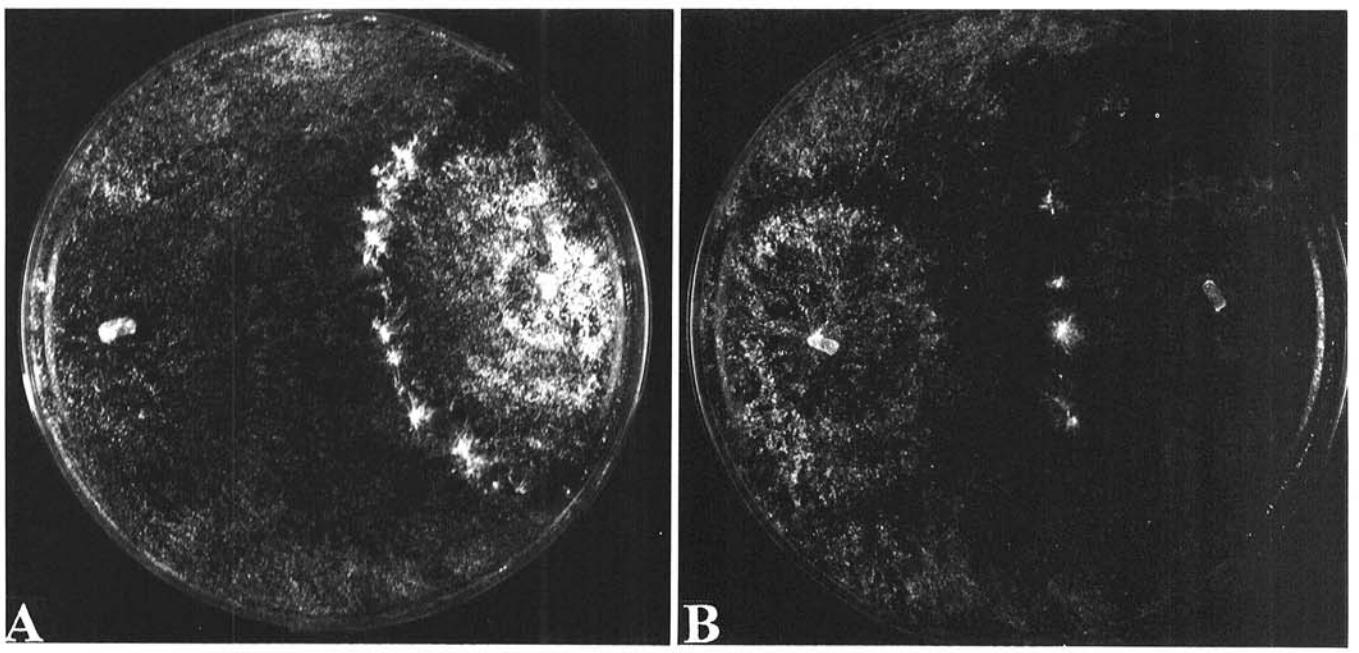

Fig. 3. Small discontinuous tuft formed between a heterokaryon and a homokaryon on potato-dextrose charcoal agar. **A,** *Thanatephorus cucumeris* parent field isolate 1R4 and its derived single-spore isolate (SSI) *32. **B,** In vitro-generated heterokaryon of SSIs *15 × *5 and its constituent homokaryon *15.

multiple vegetative incompatibility genes are responsible for incompatibility responses (6,15). Genes present at the vegetative incompatibility loci are mostly involved in allelic interactions, but they also can be involved in nonallelic interactions (15,23). Although vegetative incompatibility in basidiomycetes has not been studied as extensively as in ascomycetes, the existence of loci that govern vegetative compatibility and differ from mating-type loci have been described in basidiomycetes (15).

In our experiments, we observed that vegetative incompatibility reactions in pairings between *T. cucumeris* AG-1 homokaryons was not reproducible. This variation affected both the occurrence of killing and the extent of killing. It is not likely that this was due to variation in experimental conditions between experiments, because the same media, isolate stocks, and environmental conditions were used. Furthermore, variation in the killing reaction was observed even within experiments. Variation in severity of the killing reaction also was observed by McKenzie et al. (27) in pairings of mutant homokaryons. The fact that lysis of anastomosed cells is not always reproducible and generally seems to be a gradual process hints at the possibility that vegetative incompatibility in *T. cucumeris* is dependent on a delicate balance between the components involved. This is best expressed in genetic terms as "variable penetrance and expressivity," i.e., expression of the phenotype associated with a certain genotype can be modified by the rest of the genome (modifiers, epistatic genes, or suppressors) and by environmental factors. These findings indicate that vegetative compatibility in *T. cucumeris* is a complex mechanism that, as in other higher fungi, could involve several genetic loci.

The formation of a heterokaryon by homokaryons that are vegetatively incompatible seems contradictory, because hyphal cells die after anastomosis due to vegetative incompatibility. Our observation that a heterokaryon can be formed despite a vegetative incompatibility reaction can be explained by the hypothesis that the killing reaction is not immediate: it is a progressive reaction that is expressed at different levels of intensity in different pairings. If the killing reaction is not immediate, but is initiated some time after the different nuclei from both parent homokaryons have come together, silencing of recessive alleles may occur.

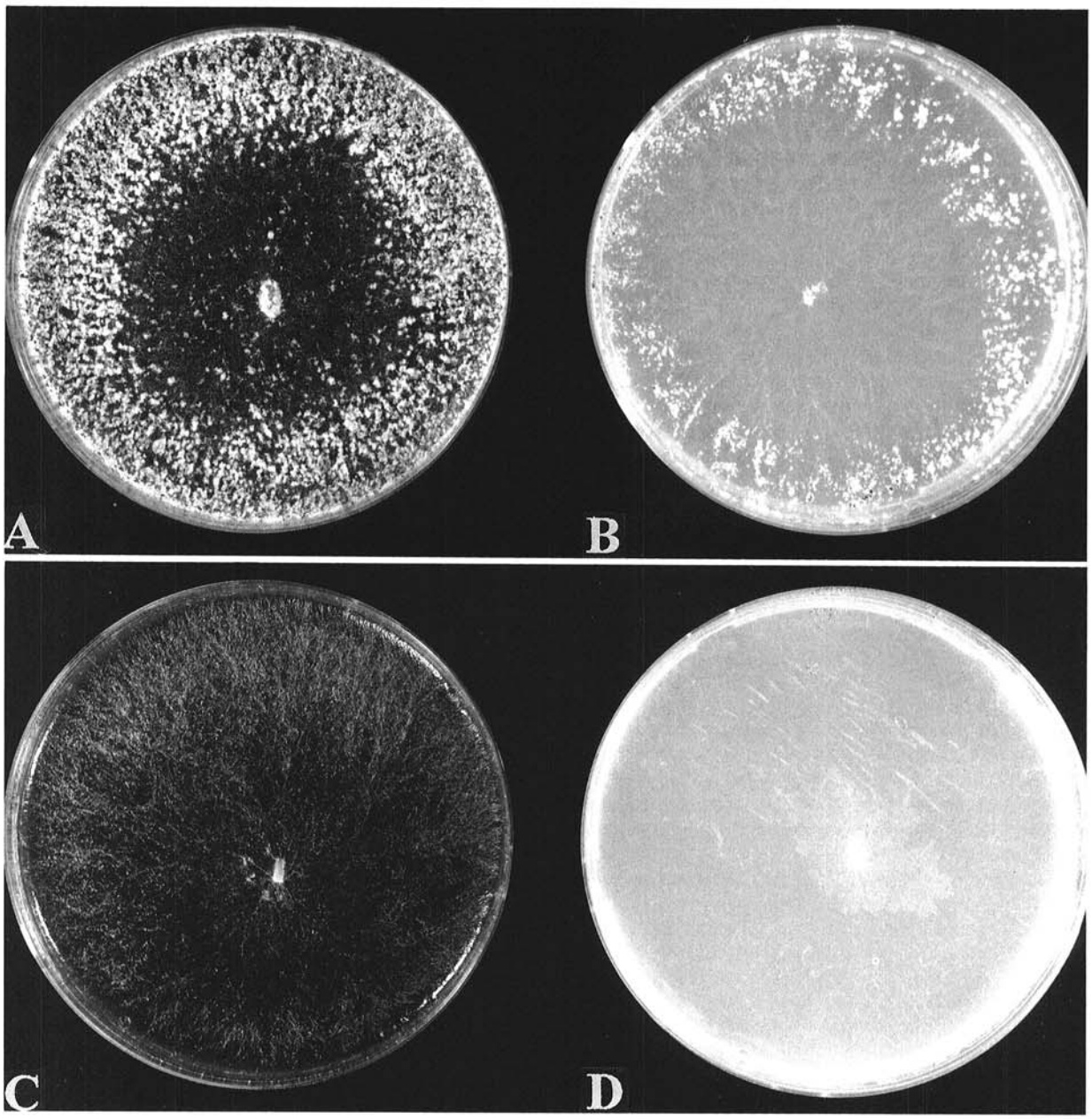

Fig. 4. Nonlysing single-spore isolate (SSI) *31 of *Thanatephorus cucumeris* on **A,** potato-dextrose charcoal agar (PDCA) and **B,** water agar plus cellophane (WA + cellophane). Spontaneously lysing SSI *12 on **C,** PDCA and **D,** WA + cellophane.

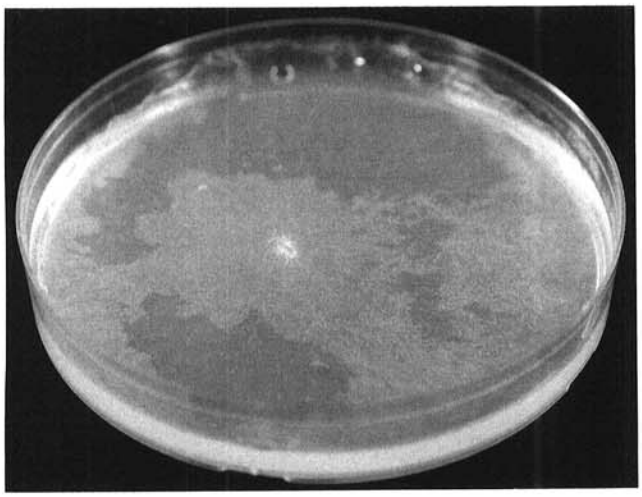

Fig. 5. Spontaneous lysing of single-spore isolate *12 of *Thanatephorus cucumeris* on water agar plus cellophane 12 days after inoculation.

In the basidiomycete *Coprinus cinereus*, Freedman and Pukkila (17) showed that silencing of heterokaryotic loci occurs in dikaryons. Silencing is caused by a genetic mechanism that detects and inactivates repeated sequences by massive cytosine methylation (17). A mechanism analogous to silencing in *Coprinus cinereus* could take place in *T. cucumeris*. This mechanism would allow the newly formed heterokaryon to escape the killing reaction. Experiments done by McKenzie et al. (27) with mutant siblings showed that despite killing reactions, heterokaryons could be formed at 25°C but not at 5°C. This indicates that escape from killing is dependent on growth rate and metabolic activity.

Alternatively, the formation of heterokaryons despite the occurrence of vegetative incompatibility reactions can be explained by the "override" hypothesis of Rayner et al. (32). In the formation of heterokaryons, somatic structures are partially or wholly involved during the mating process. The override hypothesis states that the degree to which acceptance is achieved is determined by the extent to which rejection is expressed. Acceptance involves override of rejection or vice versa, i.e., formation of a heterokaryon when homokaryons from different mating types are paired (accep-

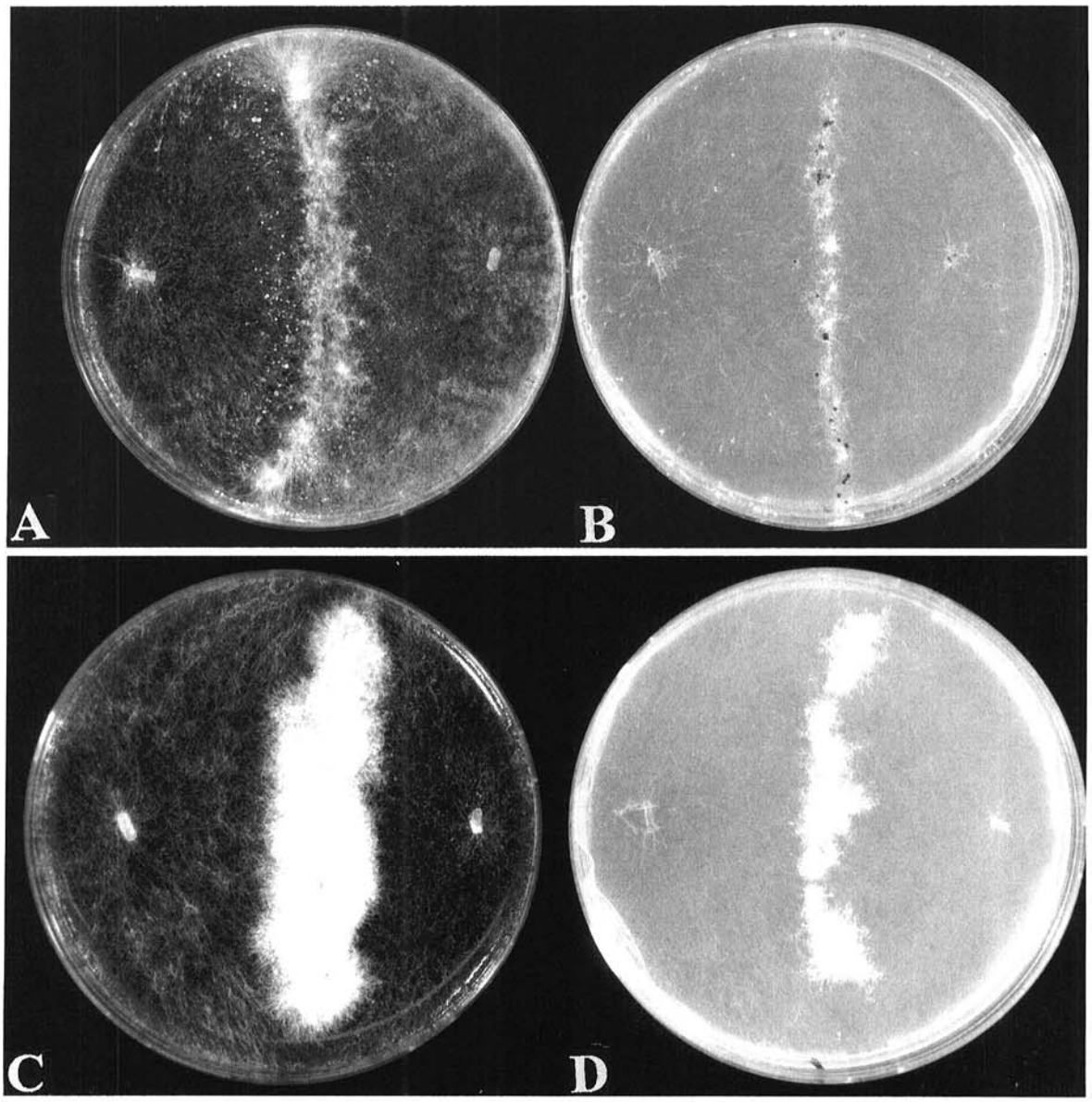

Fig. 6. Fibrous tufts formed between nonlysing single-spore isolates (SSIs) *1 and *22 of *Thanatephorus cucumeris* on **A,** potato-dextrose charcoal agar (PDCA) and **B,** water agar plus cellophane (WA + cellophane), and compact tufts formed between spontaneously lysing SSIs *3 and *12 on **C,** PDCA and **D,** WA + cellophane.

tance) is achieved only when the expression of vegetative incompatibility is lowered below certain levels. The two explanations are not necessarily mutually exclusive because the mechanism that determines override is unknown. It could be that initial override is only stable because heterozygous genes are subsequently silenced.

Small discrete fluffy tufts were formed in some pairings of the heterokaryotic field isolate 1R4 with its homokaryotic progeny as well as in pairings of in vitro-generated heterokaryons with their homokaryotic parents. This formation of discrete fluffy tufts might be associated with the formation of heterokaryons. The heterokaryotization of homokaryotic mycelium by heterokaryons of the same species implies the crossing of a heterokaryon with a homokaryon, in which migration of a nucleus can take place in one direction from heterokaryon to homokaryon. This phenomenon, commonly known as the Buller phenomenon, has been reported for a number of basidiomycetes (11) and may occur in *T. cucumeris*. This has been suggested previously by Bolkan and Butler (7) who reported that heterokaryotic field isolates and homokaryons might interact to produce new heterokaryons when paired. The fact that the tufts that were formed when a heterokaryon and a homokaryon were paired were very small and were produced only in a few of the pairings indicates that heterokaryotic-homokaryotic mating, if this is the case, does not take place extensively.

Formal experimental proof of heterokaryotic-homokaryotic mating is still lacking. Nevertheless, the implications of the possible existence of this process in *T. cucumeris* may be tremendous. The exchange of genetic material and subsequent recombination between isolates in nature would be widely increased. Recently, Naito (28) has reported the wide occurrence of sporulation of different AGs in nature. In addition to their role in infection, spores can spread and develop into homokaryotic colonies. These, in turn, can be heterokaryotized by anastomosis with another homokaryon or, if the Buller phenomenon takes place, by anastomosis with a heterokaryotic colony from the same AG. This would enhance drastically the possibility of generating genetic variation, because heterokaryotic field isolates are omnipresent. The ultimate implication of this phenomenon would be a widening of the outbreeding potential of *T. cucumeris*, with all the consequences this would have for the fungus in terms of recombination and adaptation, consequently affecting the genetic population structure and, thereby, the ecology and pathology of *T. cucumeris*.

Spontaneous lysis of the SSIs. Massive spontaneous lysis was observed for the first time in pairings between SSIs. At first, this phenomenon seemed similar to the killing properties possessed by certain strains of *Ustilago maydis* (21), caused by double-stranded RNA. Also, in *Podospora* and *Neurospora*, senescence is caused by the presence of an extrachromosomal element, a mitochondrial plasmid (24), that can be transferred horizontally (14). In *T. Cucumeris*, spontaneous lysis is a phenomenon inherent in a subset of the isolates and is not transmitted from one isolate to another. Additional evidence rejecting the possibility of the involvement of plasmid-like elements was obtained by analysis of total DNA on agarose gels and cesium chloride gradients, which did not reveal a plasmid band in any of the tested SSIs nor in the parent.

The spontaneous lysis phenomenon has not been reported before in *T. cucumeris*. That it appears in an almost 1:1 ratio among the sibling progeny of the heterokaryotic field isolate 1R4 suggests that this trait is controlled by a nuclear gene. Spontaneous lysis seems to be a recessive trait, because the heterokaryotic parent does not show spontaneous lysis. This is supported by the observation that in pairings of spontaneously lysing with nonlysing isolates, all in vitro-generated heterokaryons were nonlysing. As expected, pairings between spontaneously lysing isolates produced only spontaneously lysing heterokaryons, and pairings between nonlysing isolates produced only nonlysing heterokaryons.

It is not clear what causes spontaneous lysis. It could be that spontaneously lysing isolates are auxotrophs for some nutrient. On rich media, such as FNA or PMDA, all isolates grew very densely and formed sclerotia. On poor media, spontaneously lysing isolates were able to grow for a time with the reserves accumulated on stock FNA plates, and lysis would then be caused by starvation.

The morphology of the tufts formed when homokaryons from different mating types were paired correlates with the spontaneous lysis phenotype. Fibrous tufts were formed when nonlysing isolates or a spontaneously lysing isolate with a nonlysing isolate were paired, and compact tufts were formed only when spontaneously lysing isolates were paired. The formation of fibrous tufts was accompanied by the formation of sclerotia on top of these tufts. Because sclerotia are the survival structures of *T. cucumeris*, sclerotium formation on top of the tuft indicates that *T. cucumeris* is rapidly preserving the newly formed heterokaryon for survival. Sclerotia are never formed on the compact tufts, indicating that the spontaneous lysis allele not only causes mycelial lysis but also prevents the survival of the reconstituted heterokaryon.

These observations point to the potential advantage presented by heterokaryotic hyphae of *T. cucumeris* over homokaryotic hyphae. Indeed, as far as we know, field isolates are heterokaryons. The heterokaryotic state supplies the fungus with better possibilities for recombination, outbreeding, and adaptation. Nuclear complementation can occur in a heterokaryon, neutralizing potentially harmful alleles. It could be argued that many fungal species live successfully as homokaryons, but it is necessary to consider that *T. cucumeris* is a predominantly ectotrophic soilborne fungus. Its life cycle occurs mainly outside the protective environment of the plant. Endotropic homokaryotic fungal pathogens do not suffer

TABLE 2. Grouping of single-spore isolates (SSIs) of *Thanatephorus cucumeris* according to mating type and lysis behavior

Mating type	Lysis	SSIs[z]
M1	Nonlysing	*1, *2, *10, *13, *19, *20, *25, *27, *30, *39, *40, *41, *42, *43, (*23)
	Spontaneous	*3, *5, *14, *18, *26, *37, (*23)
M2	Nonlysing	*6, *15, *17, *22, *24, *31, *32, *33, *34, *35, *36, *38
	Spontaneous	*7, *8, *9, *11, *12, *16, *21, *28, *29, *44, *45, *46

[z] The SSIs used for heterokaryon formation are underlined. SSI *23, in parentheses, showed both cell lysis and very weak formation of sclerotia.

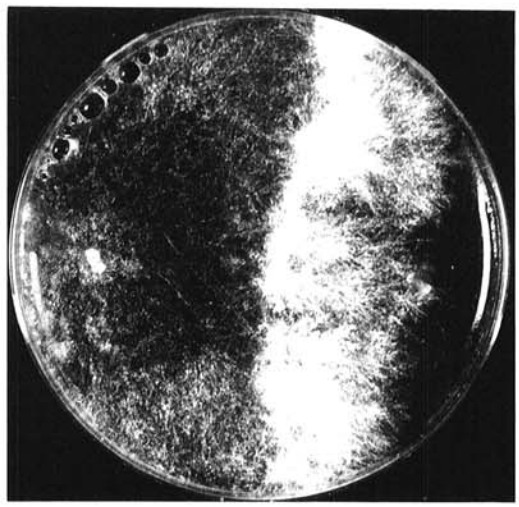

Fig. 7. Fibrous tuft formed on potato-dextrose charcoal agar (PDCA) between nonlysing single-spore isolate (SSI) *15 and spontaneously lysing SSI *3 of *Thanatephorus cucumeris*. The generated tuft overgrew spontaneously lysing SSI *3.

such high pressure from the highly variable outer environment. The presence of different nuclei in the heterokaryons of *T. cucumeris* enlarges the possibilities for ecological adaptation and survival of this fungus.

LITERATURE CITED

1. Adams, G. C. 1988. *Thanatephorus cucumeris* (*Rhizoctonia solani*), a species complex of wide host range. Pages 535-552 in: Advances in Plant Pathology. D. S. Ingram and P. H. Williams, eds. Vol. 6, Genetics of Plant Pathogenic Fungi. G. S. Sidhu, ed. Academic Press, London.
2. Adams, G. C., and Butler, E. E. 1982. A reinterpretation of the sexuality of *Thanatephorus cucumeris* anastomosis group four. Mycologia 74:793-800.
3. Anderson, N. A. 1982. The genetics and pathology of *Rhizoctonia solani*. Annu. Rev. Phytopathol. 20:329-347.
4. Anderson, N. A. 1983. Variation and heterokaryosis in *Rhizoctonia solani*. Pages 367-382 in: The Ecology and Physiology of the Fungal Mycelium. D. H. Jennings and A. D. M. Rayner, eds. Cambridge University Press, Cambridge.
5. Anderson, N. A., Stretton, H. M., Groth, J. V., and Flentje, N. T. 1972. Genetics of heterokaryosis in *Thanatephorus cucumeris*. Phytopathology 62:1057-1065.
6. Bégueret, J., Turcq, B., and Clavé, C. 1994. Vegetative incompatibility in filamentous fungi: *het* genes begin to talk. Trends Genet. 10:441-446.
7. Bolkan, H. A., and Butler, E. E. 1973. Studies on heterokaryosis and virulence of *Rhizoctonia solani*. Phytopathology 64:513-522.
8. Butler, E. E., and Bolkan, H. A. 1973. A medium for heterokaryon formation in *Rhizoctonia solani*. Phytopathology 63:542-543.
9. Carling, D. E. Grouping in *Rhizoctonia solani* by hyphal anastomosis reaction. In: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Control. B. Sneh, S. Jabaji-Hare, S. Neate, and G. Dijst, eds. Kluwer Academic Publishers, Dordrecht, Netherlands. In press.
10. Carling, D. E., Rothrock, C. S., MacNish, G. C., Sweetingham, M. W. Brainard, K. A., and Winters, S. W. 1994. Characterization of anastomosis group 11 (AG-11) of *Rhizoctonia solani*. Phytopathology 84:1387-1393.
11. Carvalho, D. B., Smith, M. L., and Anderson, J. B. 1995. Genetic exchange between diploid and haploid mycelia of *Armillaria gallica*. Mycol. Res. 99:641-647.
12. Casselton, L. A., and Kües, U. 1994. Mating-type genes in homobasidiomycetes. Pages 307-321 in: The Mycota. I. Growth, Differentiation and Sexuality. J. G. H. Wessels and F. Meinhardt, eds. Springer-Verlag, Berlin.
13. Cubeta, M. A., Briones-Ortega, R., and Vilgalys, R. 1993. Reassessment of heterokaryon formation in *Rhizoctonia solani* anastomosis group 4. Mycologia 85:777-787.
14. Debets, F., Yang, X., and Griffiths, J. F. 1994. Vegetative incompatibility in *Neurospora*: Its effect on horizontal transfer of mitochondrial plasmids and senescence in natural populations. Curr. Genet. 26:113-119.
15. Esser, K., and Blaich, R. 1994. Heterogenic incompatibility in fungi. Pages 211-232 in: The Mycota. I. Growth, Differentiation and Sexuality. J. G. H. Wessels and F. Meinhardt, eds. Springer-Verlag, Berlin.
16. Exner, B., and Chilton, S. J. P. 1943. Variation in single-basidiospore cultures of *Rhizoctonia solani*. Phytopathology 33:171-174.
17. Freedman, T., and Pukkila, P. J. 1993. *De novo* methylation of repeated sequences in *Coprinus cinereus*. Genetics 135:357-366.
18. Garza-Chapa, R., and Anderson, N. A. 1966. Behavior of single-basidiospore isolates and heterokaryons of *Rhizoctonia solani* from flax. Phytopathology 56:1260-1268.
19. Glass, N. L., and Kuldau, G. A. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. Annu. Rev. Phytopathol. 30: 201-224.
20. Keijer, J., Houterman, P. M., Dullemans, A. M., and Korsman, M. G. Heterogeneity in electrophoretic karyotype within and between anastomosis groups of *Rhizoctonia solani*. Mycol. Res. In press.
21. Koltin, Y., and Day, P. R. 1976. Inheritance of killer phenotype and double-stranded RNA in *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 73:594-598.
22. Kuninaga, S., and Yokosawa, R. 1982. DNA base sequence homology in *Rhizoctonia solani* Kühn. I. Genetic relatedness within anastomosis group 1. Annu. Phytopathol. Soc. Jpn. 48:659-667.
23. Leslie, J. F. 1993. Fungal vegetative compatibility. Annu. Rev. Phytopathol. 31:127-150.
24. Marbach, K., and Stahl, U. 1994. Senescence of mycelia. Pages 195-210 in: The Mycota. I. Growth, Differentiation and Sexuality. J. G. H. Wessels and F. Meinhardt, eds. Springer-Verlag, Berlin.
25. May, G. 1988. Somatic incompatibility and individualism in the coprophilous basidiomycete *Coprinus cinereus*. Trans. Br. Mycol. Soc. 91:443-451.
26. May, G., Le Chevanton, L., and Pukkila, P. J. 1991. Molecular analysis of the *Coprinus cinereus* mating type A factor demonstrates an unexpected complex structure. Genetics 128:529-538.
27. McKenzie, A. R., Flentje, N. T., Stretton, H. M., and Mayo, M. J. 1969. Heterokaryon formation and genetic recombination within one isolate of *Thanatephorus cucumeris*. Aust. J. Biol. Sci. 22:895-904.
28. Naito, S. 1995. Basidiospore dispersal and survival. (Abstr.) Page 88 in: Proc. Int. Symp. *Rhizoctonia*. Misset Publishers, Doetinchen, Netherlands.
29. Ogoshi, A. 1976. Studies on the grouping of *Rhizoctonia solani* Kühn with hyphal anastomosis and on the perfect stages of groups. Bull. Natl. Inst. Agric. Sci. Sec. C 30:1-163.
30. Ogoshi, A. 1987. Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annu. Rev. Phytopathol. 25: 125-143.
31. Puhalla, J. E., and Carter, W. W. 1976. The role of the H locus in heterokaryosis in *Rhizoctonia solani*. Phytopathology 66:1348-1353.
32. Rayner, A. D. M., Coates, D., Ainsworth, A. M., Adams, T. J. H., Williams, E. N. D., and Todd, N. K. 1983. The biological consequences of the individualistical mycelium. Pages 509-540 in: The Ecology and Physiology of the Fungal Mycelium. D. H. Jennings and A. D. M. Rayner, eds. Cambridge University Press, Cambridge.
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.
34. Sneh, B., Burpee, L., and Ogoshi, A., eds. 1991. Identification of *Rhizoctonia* species. The American Phytopathological Society, St. Paul, MN.
35. Whitney, H. S., and Parmeter, J. R., Jr. 1963. Synthesis of heterokaryons in *Rhizoctonia solani* Kühn. Can. J. Bot. 41:879-886.
36. Yang, H. A., Tommerup, I. C., Sivasithamparam, K., and O'Brien, P. A. 1992. Heterokaryon formation with homokaryons derived from protoplasts of *Rhizoctonia solani* anastomosis group eight. Exp. Mycol. 16: 268-278.