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Characterization of *Rhizoctonia solani* AG 2 isolates causing bare patch in field grown tulips in the Netherlands

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Abstract

During a spring survey in 1991, 130 isolates of *R. solani* were collected in 25 commercial flower bulb fields from diseased plants occurring in bare patches. On the basis of hyphal fusion frequency and pathogenicity to flower bulbs, tulip isolates were provisionally assigned to AG 2-t to distinguish these isolates from AG 2-1 isolates which were non-pathogenic to bulbs. Hyphal fusion frequency of a subgroup of 7 AG 2-t isolates was highly variable when paired with 7 AG 2-1 isolates (2–75%), thus making assignment of AG 2-t isolates to AG 2-1 inconclusive. The mean hyphal fusion frequency among AG 2-t isolates was 65% (±6%) indicating AG 2-t to be a relatively homogeneous group. Hyphal fusion frequency among AG 2-1 isolates was highly variable with a mean 51% (±25%) indicating AG 2-1 to be a heterogeneous group. The optimum growth temperature for AG 2-t and AG 2-1 isolates on malt peptone agar was 20–25 °C. The host range of AG 2-t and two AG 2-1 isolates comprised tulip, iris, hyacinth and lily at both 9 and 18 °C, and cruciferous, sugarbeet and lettuce seedlings at 18 °C. Six other AG 2-1 isolates were pathogenic to cruciferous seedlings, but not to any of the bulbous crops. The tested narcissus, *Tagetes patula*, tomato, potato, wheat, leek and maize cultivars were not susceptible to AG 2-t and AG 2-1 isolates. Statistical analysis using a *proportional-odds* model revealed significant differences in aggressiveness between *R. solani* AG 2-t isolates and differences in susceptibility between tulip and iris cultivars. At 18 °C, but not at 9 °C, isolates representing AG 2-2, AG 4, AG 5 and AG BI were pathogenic to bulbous crops. In addition to bare patch causing AG 2-t isolates, other anastomosis groups may cause disease in field grown tulips. For the development of optimal crop rotation schedules, the impact of bulb rot causing isolates under field conditions needs further study.

Introduction

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a plant pathogenic fungus, causing severe damage in many agricultural and horticultural crops worldwide (Ogoshi, 1987; Sneh et al., 1991). Isolates of *R. solani* are extremely variable in cultural morphology and host range. At present, *R. solani* is considered a species complex rather than a single species (Anderson, 1982). The species complex can be subdivided by means of hyphal anastomosis reactions between isolates into more homogeneous groups called anastomosis groups (AGs). The AGs are considered to be genetically isolated (Anderson, 1982; Kuninaga, 1996). To date, 12 AGs of *R. solani* have been described (AG 1 to AG 11, plus AG BI) (Sneh et al., 1991; Carling et al., 1994). Within AGs, subgroups are distinguished according to host range, colony morphology, thiamine requirement, and biochemical and molecular characteristics (Ogoshi, 1987; Sneh et al., 1991). In the Netherlands, *R. solani* AG 1, AG 2, AG 3, AG 4, and AG 5 have been found (Loerakker and van Dreven, 1985), and AG 2, AG 4 and AG 5 have been reported in connection with 'rhizoctonia disease' in flower bulbs and bulb flowers (Boerema and Hamers, 1988). Isolates of AG 2, AG 4 and AG 5 can readily be identified as to AG using anastomosis tests. AG 2 is further subdivided according to rela-

tive hyphal fusion frequency, thiamine requirement, host range and etiology into AG 2-1, AG 2-2IIIB, AG 2-2IV (Ogoshi, 1975, 1987) and AG 2-3 (Naito and Kanematsu, 1994). *R. solani* AG 2-1 has a wide host range (Sneh et al., 1991), including tulip (Nakatomi and Kaneko, 1971). Isolates of *R. solani* causing leaf blight in tulip (Nakatomi and Kaneko, 1971) were classified as cultural type II according to Watanabe and Matsuda (1966). Later, Ogoshi (1975) assigned Watanabe and Matsuda's cultural type II isolates to AG 2-1. AG 2-2IIIB is pathogenic to *Graminaceae* (Ogoshi, 1987), gladiolus (Takano and Fujii, 1972) and various other crops (Sneh et al., 1991). AG 2-2IV is mainly pathogenic to *Chenopodiaceae*.

Among early reports on rhizoctonia disease there is mention of bulb and stem rot of tulips (Van Poeteren, 1928; MacLean, 1948), neck and bulb rot in iris (Sonderman and MacLean, 1949), and reduced growth, root rot (Bald et al., 1955) and yellow discoloration (McWorther, 1957) in glasshouse grown lily in the US. Van Poeteren (1928) described bare patches caused by *R. solani* in both field grown and glasshouse grown tulips in California. In the Netherlands, bulb and stem rot of tulips was first reported in 1952 by Jaarsveld (1952) and since then rhizoctonia disease has become an increasing problem in the Dutch bulb growing areas (Muller, 1969). Rhizoctonia disease in field grown tulips frequently occurs as bare patches, similarly to rhizoctonia bare patch disease of cereals (MacNish and Neate, 1996). Bare patches in tulip develop at low soil temperatures when the sprouts grow through the soil. Doornik (1981) described the temperature dependency of *R. solani* isolates infecting sprouts of tulip, hyacinth, lily, iris and anemone in glasshouse experiments as an intrinsic characteristic of the isolates. Her 'warmth preferring' isolates, infecting bulb crops only at $\geq$13 °C, and 'cold preferring' isolates, infecting bulb crops mainly at $\leq$13 °C, later were identified as different AGs and assigned to AG 4 and to AG 2 respectively (Loerakker and Van Dreven, 1985). In addition to these isolates of AG 2 and AG 4, isolates of AG 2-1 and AG 5 have been associated with rhizoctonia disease in flower bulbs (Boerema and Hamers, 1988). However, the subgrouping of cold preferring AG 2 isolates (Doornik, 1981) remained unclear.

In order to develop crop protection strategies in field grown flower bulbs based on the ecology of the pathogen including e.g. crop rotation, a precise characterization of *R. solani* isolates causing bare patch in tulip is essential. The present paper intends to characterize *R. solani* isolates causing bare patch in field grown tulip using hyphal anastomosis, thiamine requirement, growth characteristics on agar and host range.

Materials and methods

Pathogen isolation and collection

From April to June 1991, plants with *Rhizoctonia* symptoms were collected from bare patches in 26 bulb flower fields throughout the Netherlands. Pieces of infected bulb, stem, and leaf tissue were surface sterilized in 1% sodium-hypochlorite for 30 sec, rinsed once in sterile tap water for 1 min, plated on 1.5% tap water agar amended with 250 ppm chloramphenicol and 250 ppm metalaxyl (WACM) and incubated for 2 to 3 days at room temperature. After a second transfer to WACM, isolates were transferred to malt-peptone agar (MPA), containing 15 g Malt extract (Oxoid L39), 1.25 g special peptone (Oxoid L72), and 15 g agar (Oxoid L13) per litre of tapwater (Van den Boogert and Jager, 1984). Pure cultures were stored on MPA-slants at 10 °C. *R. solani* isolates obtained from bare patches in tulip and iris used in this study are listed in Table 1 and on the basis of the experimental data in this paper further referred to as AG 2-t. Isolates representing other AGs are in Table 2.

Preliminary identification of field isolates

Out of 130 field isolates, 43 were selected according to the geographical location of the production field and the site of the lesions (leaf, stem or bulb). The AG identity of these 43 isolates was determined (see AG typing section) using anastomosis tester isolates of AG 1 through AG 5 (Table 2). In addition, these 43 isolates were tested for pathogenicity on tulip cv. 'Red Riding Hood' (see pathogenicity tests on flower bulbs section). For further experiments a subset of arbitrarily chosen isolates was used.

Anastomosis group typing

Water agar coated slides were seeded with mycelial disks (5 mm in diameter) taken from the edge of growing colonies on MPA, placed on moist filter paper in large (20 cm in diameter) Petri dishes and incubated for 24-30 h at room temperature (Tu et al., 1969). Disks of field isolates and anastomosis tester isolates were spaced approximately 1 cm. When the colonies over-

Table 1. Codes, origin and thiamine requirement of *Rhizoctonia solani* AG 2-t isolates used in this study

IPO-code[1]	cultivar[2]	site of isolation	location in the Netherlands	sampling date day/month/year	thiamine requirement[3]
2tR002[4]	Red Riding Hood	stem	Noordwijkerhout	. .0575	+
2tR101	Varinas	leaf	Breezand	260491	+
2tR102	Varinas	stem	Breezand	260491	+
2tR103	Halcro	leaf	Breezand	260491	+
2tR104	Halcro	stem	Breezand	260491	n.d.
2tR105[4]	Menton	stem	Breezand	260491	+
2tR106	Menton	soil	Breezand	260491	n.d.
2tR107	Estella Rijnveld	stem	Julianadorp	260491	+
2tR108	Giant Parrot	bulb	Noordwijkerhout	260491	n.d.
2tR109	Giant Parrot	stem	Noordwijkerhout	080591	+
2tR110	Giant Parrot	soil	Noordwijkerhout	080591	n.d.
2tR111	Inzell	leaf	Noordwijkerhout	080591	+
2tR112	Inzell	soil	Noordwijkerhout	080591	n.d.
2tR113	*Iris* 'White Excelsior'	stem	Heemskerk	080591	n.d.
2tR114[4]	*Iris* 'White Excelsior'	bulb	Heemskerk	080591	+
2tR115	*Iris* 'White Excelsior'	soil	Heemskerk	080591	n.d.
2tR116	*Ixia* spp.	bulb	Heemskerk	080591	n.d.
2tR117	*Ixia* spp.	stem	Heemskerk	080591	n.d.
2tR118[4]	Leen van der Mark	leaf	Castricum	080591	+
2tR119	Leen van der Mark	stem	Castricum	080591	n.d.
2tR120	Leen van der Mark	bulb	Castricum	080591	n.d.
2tR121	Leen van der Mark	soil	Castricum	080591	n.d.
2tR122	Judith Leyster	leaf	St. Pancras	080591	n.d.
2tR123	Judith Leyster	stem	St. Pancras	080591	+
2tR124[4]	Judith Leyster	bulb	St. Pancras	080591	+
2tR125	Gander	leaf	Breezand	170591	+
2tR126	Gander	stem	Breezand	170591	n.d.
2tR127	Gander	soil	Breezand	170591	+
2tR128[4]	Pink Supreme	leaf	Anna Paulowna	170591	+
2tR129	Plaisir	leaf	Anna Paulowna	170591	n.d.
2tR130	Polo	leaf	Lisse	120691	+
2tR133	Hurts Delight	stem	De Zilk	050691	n.d.
2tR134	Hurts Delight	bulb	De Zilk	050691	+
2tR135[4]	Red Riding Hood	leaf	De Zilk	050691	+
2tR136	Plaisir	stem	De Zilk	050691	n.d.
2tR137	Ali Baba	bulb	De Zilk	050691	n.d.
2tR138	Red Riding Hood	leaf	Den Helder	120691	+
2tR139[4]	Red Riding Hood	bulb	Den Helder	120691	+
2tR140	Pinoccio	bulb	Anna Paulowna	120691	+
2tR142[4]	Red Riding Hood	leaf	Lisse	120691	+
2tR143	Red Riding Hood	bulb	Lisse	120691	n.d.
2tR144[4]	Fashion	stem	Lisse	120691	+
2tR145	*Hyacinthus* spp.	bulb	Lisse	160791	+
2tR146	*Hyacinthus* spp.	stem	Lisse	160791	+

[1] All isolates were collected by JHM Schneider, except for isolate 2tR002 which was provided by the Bulb Research Centre, LBO (Lisse, the Netherlands), original isolate designation T8 [Doornik, 1981], ATCC 56614.
[2] Tulip cultivars unless otherwise mentioned.
[3] +: Thiamine prototrophic; n.d.: not determined.
[4] Isolates deposited at the Centraalbureau voor Schimmelcultures (CBS), Baarn, the Netherlands as CBS 198.97 to 207.97, respectively.

Table 2. Codes and origin of *Rhizoctonia solani* AG 1 to AG 6 and AG BI isolates used in this study

IPO-code	AG typing	Host	Origin	Original isolate designation	Source[1]
01R01[2]	1-IA	rice	Japan	CS-KA	Ogoshi
01R02[2]	1-IB	sugar beet	Japan	B-19	Ogoshi
01R03[2]	1-IC	sugar beet	Japan	BV-7	Ogoshi
21R01[2]	2-1	pea	Japan	PS-4	Ogoshi
21R06	2-1	tulip	Japan	TG-1	Ogoshi
21R11	2-1	cauliflower	the Netherlands	PD 80/710	PD
21R12	2-1	cauliflower	the Netherlands	PD 81/228	PD
21R14	2-1	cauliflower	the Netherlands	PD 86/723	PD
21R21	2-1	cauliflower	the Netherlands	PD 86/748	PD
21R41	2-1	swede	the Netherlands	PD 81/130	PD
21R51	2-1	oil-seed rape	the Netherlands	PD 80/664	PD
21R61	2-1	lily	the Netherlands	PD 83/866	PD
21R71	2-1	turnip rape	the Netherlands	PD 83/810	PD
21R81	2-1	lettuce	the Netherlands	PD 83/909	PD
21R91	2-1	leek	the Netherlands	PD 83/303	PD
22R01[2]	2-2IIIB	mat rush	Japan	C-96	ATCC 76124
22R02[2]	2-2IV	sugar beet	Japan	RI-64	ATCC 76125
22R11	2-2	sugar beet	the Netherlands	PD 85/904	PD
03R01[2]	3	potato	Japan	ST-11-6	Ogoshi
03R03	3	potato	the Netherlands	36 AB65	Jager
03R04	3	potato	the Netherlands	09 ABa	Jager
03R05	3	potato	the Netherlands	05 AHa	Jager
04R01[2]	4	peanut	Japan	AH-1	Ogoshi
04R08	4	iris	Israel	Iyot	LBO
04R09	4	lettuce	the Netherlands	S1	LBO
04R10	4	lettuce	the Netherlands	S2	LBO
04R11	4	bean	the Netherlands	PD 82/576	PD
04R22	4	iris	the Netherlands	I7	LBO
04R61	4	spinach	the Netherlands	PD 83/418	PD
04R70	4	anemone	the Netherlands	PD 84/659	PD
04R71	4	cucumber	the Netherlands	PD 84/762	PD
05R01[2]	5	soybean	Japan	GM-10	Ogoshi
05R06	5	lily	the Netherlands	PD 82/741	PD
05R21	5	maize	the Netherlands	PD 83/481	PD
05R31	5	soil	the Netherlands	PD 84/865	PD
06R01[2]	6 HG-1	soil	Japan	OMT-1-1	Ogoshi
BIR01[2]	BI	soil	Japan	Ts-2-4	Ogoshi

[1] PD: Plant Protection Service, Wageningen, the Netherlands. LBO: Bulb Research Centre, Lisse, the Netherlands.

[2] Anastomosis tester isolates [Sneh et al., 1991].

lapped the area was examined microscopically (100x) for hyphal anastomosis. AG typing was scored positively (confirmed at 400x) when 5 or more hyphal anastomosis points were observed (Carling et al., 1987; Carling, 1996). Anastomosis subgrouping within AG 2 was determined on the basis of hyphal fusion frequency (FF) (Ogoshi, 1975; Carling and Sumner, 1992) using seven AG 2-t isolates and seven AG 2-1 isolates. Average values of FF per five microscopic fields in three pairings were calculated.

Thiamine requirement

Mycelial disks (5 mm in diameter) of 25 arbitrarily chosen AG 2-t isolates (Table 1) were transferred from the edge of actively growing cultures on Czapek-Dok agar to Petri dishes (9 cm in diameter) containing Czapek-Dok liquid medium without (A) or with (B) 10^{-5} M thiamine-hydrochloride. Anastomosis tester isolates of AG 2-1, AG 2-2, AG 3 and AG 5 (Table 2) were included for comparison. Observations on growth and pigmentation were made after 14 days of incubation at 25 °C. (Ogoshi and Ui, 1979; Rovira et al., 1986). Mycelial dry weights were determined after drying overnight at 110 °C. A value of the B/A ratio less than 1.5 indicates the prototrophic nature of the isolate (Carling et al., 1987).

Growth characteristics on malt peptone agar

The average colony growth (A.C.G.) of 11 arbitrarily chosen isolates of AG 2-t (2tR002, 2tR105, 2tR114, 2tR118, 2tR123, 2tR124, 2tR128, 2tR135, 2tR138, 2tR139, 2tR144) was compared with the A.C.G. of nine isolates of AG 2-1 (21R01, 21R11, 21R12, 21R14, 21R21, 21R41, 21R51, 21R71, 21R91), and seven AG 4 isolates (4R08, 4R09, 4R10, 4R22, 4R61, 4R70, 4R71). Mycelial disks (5 mm in diameter) from the edge of actively growing cultures on MPA were placed in the centre of 9 cm Petri dishes containing MPA and incubated at 5, 10, 15, 20, 25, 30, 35, and 40 °C. Two Petri dishes per isolate were seeded. The average colony diameter was assessed at 8 regularly spaced points per colony per isolate every other day until the colony had reached the edge of the plate. The average colony growth (mm day^{-1}) per AG was plotted against temperature.

Pathogenicity tests on flower bulbs

Tulipa cvs Red Riding Hood, Apeldoorn and Gander, *Iris* cvs Blue Magic, White Excelsior and prof. Blaauw, *Hyacinthus* cvs Pink Pearl and Jan Bos, *Narcissus* cv. Tête-à-Tête and *Lilium* cv. Enchantment were obtained from commercial farmers. The three tulip cvs are classified in three taxonomically different groups (Classified List, 1987). According to farmers experiences, tulip cultivars differ in susceptibility to *R. solani* (Van Keulen and Van Aartrijk, 1993). Tulips 'Red Riding Hood' and 'Gander' are more susceptible than 'Apeldoorn'. Differences in cultivar susceptibility for *R. solani* have not been reported for iris, hyacinth, lily and narcissus. According to farmers experiences, all cultivars of iris, hyacinth and lily are to some extent susceptible to *R. solani*, whereas cultivars of narcissus are not. For forcing, tulip bulbs were stored at 17 °C and pre-treated at 2 °C in a temperature controlled storage room during 10 weeks before planting in the glasshouse. Iris bulbs were stored at 30 °C, disinfected in 4% (v/v) formalin for 15 min to eliminate external contamination and pre-treated for 6 weeks at 17 °C in a temperature controlled storage room to break dormancy, and planted in the glasshouse. Hyacinth and narcissus bulbs were stored at 30 °C and pre-treated for 6 weeks at 5–9 °C in a temperature controlled storage room. Prior to planting hyacinth and narcissus were disinfected in 4% (v/v) formalin to eliminate surface contamination. Lily bulbs were covered with a peaty soil and stored at −2 °C until use. After storage, lily bulbs were planted immediately in the glasshouse.

The virulences of eleven AG 2-t isolates to tulip, iris, hyacinths and lily were compared with the virulences of some isolates belonging to AG 2-1, AG 2-2, AG 3, AG 4, and AG 5 in glasshouse tests. The experiments were conducted simultaneously at two temperatures, 9 and 18 °C, favourable for pathogenicity of AG 2-t and AG 4, respectively (Doornik, 1981). Three bulbs were planted per 1.5 l pot filled with an unsterilised 2:1 sand:potting soil mixture and inoculated by placing two oat kernels colonized by the fungus onto the neck of each bulb (Doornik, 1981). Isolates of *R. solani* had been grown on sterilised oat kernels at 20 °C for two to three weeks. In control pots, sterilised non-infested oat kernels were used. After six weeks, tulip and hyacinth plants were evaluated for disease symptoms on the leaves and iris plants for disease symptoms on the bulb. Disease symptoms per plant were classified in one of five qualitative disease classes 0: healthy; 1: symptoms unclear; 2: slightly infected; 3; moderately infected; 4: heavily infected or dead. Isolates causing disease classes 0 and 1 were considered non-pathogenic under the prevailing conditions. For lily, pathogenicity of AG 2-t isolates was expressed

as the mean percentage reduction of stem length per pot. The experimental design was a complete randomized block with four replications. Pathogenicity tests using all isolates, tulip 'Red Riding Hood', and iris 'Blue Magic' were repeated in a second experiment.

Pathogenicity tests on cruciferous and other seedlings

AG 2-t and AG 2-1 isolates were screened for their ability to cause disease in cruciferous seedlings in glasshouse tests at 18 °C. Seedlings of cauliflower (*Brassica oleracea* var. *botrytis*) cv. Oberon, fodder radish (*Raphanus sativus* ssp. *oleiferus*) cv. Nemex, white mustard (*Sinapis alba*) cv. Maxi, oil-seed rape (*B. napus* spp. *oleifera*) cv. Jet Neuf, and swede (*B. napus* var. *napobrassica*) cv. Friese Gele, lettuce (*Lactuca sativa*) cv. Petty, leek (*Allium porrum*) cv. Joland, sugarbeet (*Beta vulgaris*) cv. Hilde, potato (*Solanum tuberosum*) cv. Bildstar, tomato (*Lycopersicon esculentum*) cv. Moneymaker and *Tagetes patula* were tested. After germination, 8 seedlings were planted per pot (400 ml). Soil and inoculum were the same as used for the flower bulb tests. One week after transplanting, pots were infested with *R. solani*. Two oat kernels colonized with *R. solani* were placed in the centre of each pot. Four weeks after infestation, the number of healthy and diseased plants was evaluated. The experimental design was a randomized complete block with four replicates. In an additional experiment the pathogenicity tests of AG 2-t isolates on cauliflower and fodder radish were repeated and compared with the pathogenicity of AG 2-t isolates to wheat (*Triticum aestivum*) cv. Flevina, maize (*Zea mays*) cv. Brutus, and sugarbeet cv. Accord.

Statistical analysis

Significance of differences between variances of mean FF's of AG pairings was tested using a F-test (Sokal and Rohlf, 1981). Mean FF's of AG pairings were compared using an approximate t-test, considering unequal variances and unequal sample sizes (Sokal and Rohlf, 1981).

Differences in virulence and cultivar susceptibility to AG 2-t were analyzed using a *proportional-odds* model (McCullagh, 1980; McCullagh and Nelder, 1989). The model quantifies the effect of the disease rating by the construction of an underlying continuous scale on which the distribution for each isolate-cultivar combination (treatment) is logistic with standard deviation $\pi/\sqrt{3}$. For the five qualitative disease classes, four unknown cut-points provide a quantification of the differences between successive classes on the underlying scale. The model describes the relationship between numbers of observations up to a particular class. The model has the form $\ln(\gamma_{ij}/(1-\gamma_{ij})) = \theta_j - \mu_i$ where γ_{ij} is the j^{th} cumulative probability for the i^{th} treatment. Or, γ_{ij} is the probability that the response for a randomly chosen bulb falls in class j or lower. The θ_j's are the cutpoints and μ_i stands for the mean of treatment i on the underlying scale. As the mean on the underlying scale increases, the probability for a bulb being rated into the higher classes also increases. Treatment effects were assessed using differences between treatment means on the underlying scale. For the mean μ_i a linear model was used with replication, isolate and host and isolate $\times$ cultivar interactions as explanatory variables. All effects were taken as fixed except the interactions. The interactions were taken as random to circumvent estimation problems for host-isolate combinations with all observations in an outer class. Dependence between observations on bulbs within pots was investigated by incorporating an additional random effect for differences between pots in the linear model. The random effects for interactions and pot are assumed to be independently and normally distributed. Estimates of cut-points, variance components and treatment effects were obtained by the Iteratively Reweighted Restricted Maximum Likelihood Method (IRREML, Engel and Keen, 1994; Keen, 1994) using the statistical programme Genstat 5 (Genstat 5 Committee, 1993). Fixed effects were tested using Wald-tests (Cox and Hinkley, 1974). Pairwise differences between treatment means on the underlying scale were tested using a normal approximation.

For statistical analysis of the pathogenicity of AGs to lily, differences in aggressiveness of isolates within an AG were compared to the control using least significant differences (LSD). Differences in aggressiveness of isolates to cruciferous seedlings were compared to the control per AG and per cultivar using LSD values. Differences between average colony growth rates per AG per temperature were compared using LSD values.

Results

Anastomosis group typing

Hyphae of all 43 selected AG 2-t isolates (Table 1) anastomosed with the AG 2 tester isolates (data not shown). A subset of seven arbitrarily chosen AG 2-t

isolates was tested for subgrouping within AG 2. Hyphal fusion frequency (FF) of these AG 2-t isolates with AG 2-2IIIB and AG 2-2IV tester isolates was consistently less than 30% (data not shown). The average FF of these seven AG 2-t isolates with the Japanese tester isolate (21R01) varied from 9–56%, and from 2–62% when paired with Dutch AG 2-1 isolates (Table 3). Isolate 21R06, originating from tulip in Japan, fused in high frequency with AG 2-t isolates and AG 2-1 isolates, except for 21R81 (low frequency). The average hyphal fusion frequency among the seven tested AG 2-t isolates was over 50%, except for 2tR144 with 2tR105 (41%). The average FF among AG 2-1 isolates was also over 50%, except for isolate 21R81, which fused in high frequency with the tester isolate 21R01 and in self anastomosis, but in low frequency with all other AG 2-1 isolates. The average FF of AG 2-t with AG 2-t isolates varied from 41–74%. The average FF of AG 2-t with AG 2-1 isolates varied from 2–75%, and the average FF of AG 2-1 with AG 2-1 varied from 3–77%.

The mean FF, pooled average FF per AG pairing, of AG 2-t∗2-t (65%) was significantly different from the mean FF of AG 2-t∗2-1 (34%; t-test, $P < 0.01$) and from the mean of AG 2-1∗AG 2-1 (51%; t-test, $P < 0.01$). The variance of the mean FF of AG 2-t∗2-t (38) was significantly different from the variance of the mean FF of AG 2-t∗AG 2-1 (440) and from the variance of the mean FF AG 2-1∗AG 2-1 (601; F-test, $P < 0.01$). The mean FF's of AG 2-t∗AG 2-1 (34%) and AG 2-1∗2-1 (51%), were significantly different (t-test, $P < 0.01$), whereas their variances, 440 and 601 respectively, were not significantly different (F-test, $P > 0.05$) (Table 3).

Thiamine requirement

The 25 tested AG 2-t isolates (Table 1), and anastomosis tester isolates of AG 2-1 and AG 3 grew equally well on both Czapek-Dok liquid medium with (B) or without (A) thiamine (B/A ratio 0.8–1.4) and developed a brown pigmentation of the hyphae, indicating thiamine prototrophy of AG 2-t isolates. Anastomosis tester isolates of AG 2-2 and AG 5 were confirmed to be thiamine auxotrophic.

Growth characteristics on agar

Average growth curves of AG 2-t and AG 2-1 isolates were similar, but different from AG 4 (Figure 1). Optimum growth for AG 2-t and AG 2-1 isolates occurred at 20–25 °C (12 mm day^{-1}), and for AG 4 isolates at 25–30 °C (18 mm day^{-1}). At 5 °C, isolates of AG 2-t and AG 4 grew significantly ($P < 0.01$) slower (0.4 and 0.5 mm day^{-1} respectively) than AG 2-1 isolates (2.1 mm day^{-1}). At 35 °C AG 2 isolates did not grow, whereas AG 4 isolates were still capable of growth. None of the isolates of any AG grew at 40 °C.

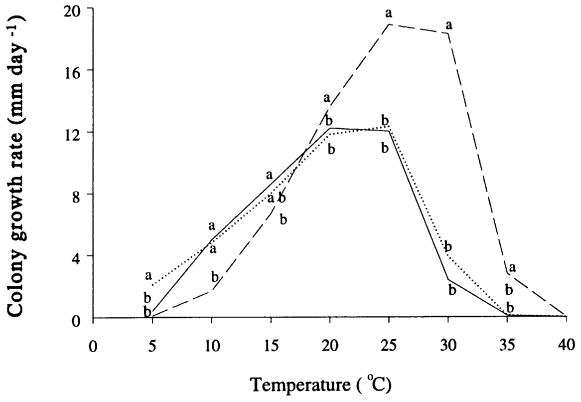

Figure 1. Average colony growth rate of AG 2-t (—), AG 2-1 (·····) and AG 4 (- - -) on Malt-Peptone Agar at 8 temperatures. Averages per temperature followed by the same letter are not significantly different ($P < 0.01$).

Pathogenicity tests on flower bulbs

In preliminary experiments, all 44 isolates listed in Table 1 were pathogenic to tulip cv. Red Riding Hood. For further research on the host range, reported in this paper, eleven arbitrarily chosen AG 2-t isolates were used.

Statistical data analysis using a *proportional-odds* model was done for combinations of AG 2-t isolates with tulip and iris cultivars separately per species and per temperature. First it was investigated whether interactions occurred between isolates and cultivars by fitting a mixed model with fixed effects for isolates and cultivars and random effects for interactions and for differences between pots. Fitting of the mixed model showed only a substantial component of variance for interaction for tulip at 9 °C, which was accounted for by the isolate/cultivar combination 2tR114/Red Riding Hood (data not shown). Excluding this combination from the analysis resulted in non-significant interactions effects. Therefore, in further data analysis the interactions were dropped from the model and a mixed model was used with fixed effects for isolate and cultivar and a random effect for pot

Table 3. Average hyphal fusion frequency (FF, in %) of AG 2-t isolates with AG 2-1 isolates (upper left) and means, standard deviations (s.d.) and variances summarized per pairing of AG's (lower right)[1]

AG		2-t							2-1						
	isolate	2tR105	2tR114	2tR118	2tR123	2tR138	2tR139	2tR144	21R81	21R61	21R41	21R21	21R14	21R06	21R01
2-1	21R01	38	9	26	51	56	44	38	50	65	68	58	64	65	67
	21R06	54	75	66	61	61	56	52	4	66	68	68	67	62	
	21R14	19	13	4	7	40	57	60	6	61	71	69	77		
	21R21	24	13	34	19	27	30	32	3	67	66	66			
	21R41	21	34	51	26	23	49	25	13	59	72		mean :	51	
	21R61	17	12	57	58	54	53	62	9	64			s.d. :	25	
	21R81	2	6	4	4	5	5	6	66				var. :	601	
2-t	2tR144	41	67	66	58	67	64	73							
	2tR139	66	65	67	62	74	59								
	2tR128	69	71	73	68	65									
	2tR123	70	66	70	65										
	2tR118	66	61	67		mean :	65						mean :	34	
	2tR114	64	67			s.d. :	6						s.d. :	21	
	2tR105	76				var. :	38						var. :	440	

[1] Mean FF of AG 2-t*2-1 (34%) was significantly different from mean FF of AG 2-t*2-t (65%; t-test, $P<0.01$) and from AG 2-1*2-1 (51%; t-test, $P<0.01$). Mean FF of AG 2-t*2-t (65%) was significantly different from AG 2-1*2-1 (51%; t-test, $P<0.05$). Variance of mean FF of AG 2-t*2-1 (440) was significantly different from AG 2-t*2-t (38), but not from AG 2-1*2-1 (601; F-test, $P<0.01$). Variances of mean FF 2-t*2-t (38) and AG 2-1*2-1 (601) were significantly different (F-test, $P<0.01$), whereas variances of mean FF 2-t*2-1 (440) and 2-1*2-1 (601) were not (F-test, $P>0.05$). FF's of self anastomosis were not included in the statistical comparisons.

differences. Differences in aggressiveness between isolates and differences in cultivar susceptibility were statistically tested using a normal approximation for the differences between the predicted means on the underlying scale.

At both 9 and 18 °C the tested AG 2-t isolates were highly virulent to the tulip cvs tested and less virulent to the iris cultivars (Tables 4 and 5). Only minor differences in virulence were observed between isolates. At both 9 and 18 °C, tulip cv. Apeldoorn was significantly ($P < 0.05$) less susceptible to AG 2-t isolates than cvs Gander and Red Riding Hood. At 9 °C, disease severity in iris cv. White Excelsior was significantly ($P < 0.05$) lower than in cvs Blue Magic and Prof. Blaauw. At 18 °C, cv. Prof. Blaauw was significantly ($P < 0.05$) less diseased than cvs Blue Magic and White Excelsior. Between iris cultivars, differences in susceptibility to AG 2-t isolates were in general less pronounced at 9 °C. At 18 °C, however, differences in cultivar susceptibility were less pronounced for tulip and more pronounced for iris cvs. At both temperatures, the tested tulip and iris cultivars Blue Magic and White Excelsior were also infected by AG 2-1 isolates 21R01 and 21R51, but not by the other AG 2-1 isolates.

At 18 °C, AG 2-t isolates were only mildly virulent to hyacinth cv. Jan Bos, whereas at 9 °C neither hyacinth cv. was diseased by the AG 2-t isolates tested. At 9 °C, seven AG 2-t isolates reduced the stem growth of lily cv. Enchantment significantly ($P < 0.01$) varying from 14-31%. At 18 °C, one isolate (2tR135) reduced the stem growth significantly ($P < 0.01$) with 12%. The Japanese tester isolate 21R01 was not pathogenic to lily at either temperature. In narcissus no disease symptoms developed at either temperature.

Isolates of AG 2-2, AG 3, AG 4, AG 5, AG 6 and AG BI did not cause any disease symptoms on bulbous crops at 9 °C. At 18 °C, however, isolates representing AG 2-2, AG 4, AG 5, and AG BI caused disease in tulip, iris and hyacinth cv. Jan Bos (Table 6), whereas isolates of AG 3 and AG 6 did not cause any symptoms.

The second experiment comprising all 34 isolates and tulip cv. Red Riding Hood and iris cv. Blue Magic gave the same results. Control plants remained free of *R. solani* symptoms.

Pathogenicity tests on cruciferous and other seedlings

All of the 11 AG 2-t and 10 AG 2-1 isolates tested caused damping-off in seedlings of cauliflower, fodder radish, white mustard, oil-seed rape and swede at 18 °C (Table 5). Damping-off in lettuce was variable and depended on the isolate tested. No damping-off was caused in leek, potato, tomato, sugarbeet cv. Hilde, and *Tagetes patula* in this experiment. In the second experiment the same results were obtained for cauliflower and fodder radish. In wheat cv. Flevina and maize cv. Brutus no damping-off occurred. AG 2-t isolates caused damping-off in sugarbeet cv. Accord, but the results (data not shown) were highly variable and isolate dependent. From some symptomless plants of *Tagetes*, sugarbeet, wheat, maize, potato, and tomato AG 2-t could be re-isolated.

Discussion

For the development of environmentally friendly and effective control measures understanding of the dynamics of pathogenic *R. solani* populations in the field is essential. Hence, a precise characterization and identification of isolates that cause rhizoctonia bare patch in field grown flower bulbs is a prerequisite. Variance of mean hyphal fusion frequency (FF) among AG 2-t isolates, suggested that *R. solani* AG 2-t isolates formed a relative homogeneous subgroup within AG 2 distinct from either AG 2-1 (Table 3) and AG 2-2. Variance of mean FF among *R. solani* AG 2-1 isolates indicated AG 2-1 a heterogeneous group. Due to great variability in FF it was not always possible to unambiguously distinguish individual AG 2-t isolates from AG 2-1 isolates (Table 3). In earlier reports 'cold-preferring' isolates from tulip (Doornik, 1981) were assigned to AG 2 (Loerakker and Van Dreven, 1985) and provisionally designated as AG 2-3[1]. Our FF tests (Table 3) do not support previous suggestions (Loerakker and Van Dreven, 1985; Cruickshank, 1990) of a new AG 2 subgroup on the basis of FF only. In the earlier experiments (Loerakker, unpubl.), FF was determined between two tulip isolates and seven AG 2-1 Dutch isolates, and among six tulip isolates. The definition of FF was poor at that time. FF of the two tulip isolates with the AG 2-1 isolates varied mostly from 'no reaction' to 'not frequent' and was described as 'frequent' in one pairing only. The use of only two tulip isolates in pairings with AG 2-1 isolates in combination with the generally observed high variability of FF made the assignment of these two tulip isolates to a

[1] Loerakker's provisionally designated AG-2-3 (Loerakker, pers comm.; Cruickshank, 1990) is not to be confused with soybean leaf infecting AG-2-3 isolates (Naito and Kanematsu, 1994). Soybean AG-2-3 isolates were not known when Loerakker's and our research was conducted.

Table 4. Average disease severity[1] of four flower bulbs grown in soil infested with isolates of *Rhizoctonia solani* AG 2-t and AG 2-1 for six weeks in the glasshouse at 9 °C

		leaf						bulb				S.L.[2]
		tulip				hyacinth		iris				lily
	cv.[3]	R	G	A		PP	JB	BM	PB	WE		E
AG	isolate				c.i.[4]						c.i.	
2-t	2tR002	4	4	3	abc	0	0	2	2	2	ab	69
	2tR105	4	4	3	abc	0	0	2	3	2	bc	-
	2tR114	3	4	3	n.i.	0	1	2	3	2	bc	81
	2tR118	4	3	2	a	-	-	2	2	1	a	78
	2tR123	4	4	2	abc	0	0	2	2	2	bc	-
	2tR124	4	3	2	ab	-	-	2	2	2	ab	-
	2tR128	4	3	2	ab	0	1	2	2	2	ab	86
	2tR135	4	4	3	c	0	0	3	3	2	c	79
	2tR138	4	4	3	bc	1	-	2	2	2	bc	71
	2tR139	4	3	2	abc	1	1	2	2	1	ab	79
	2tR144	4	3	2	abc	1	0	4	4	3	d	91
	c.c.[5]	a	b	c				a	a	b	$LSD_{0.01}$	11
2-1	21R01	4	4	2		0	0	3	1	1		110
	21R11	0	0	1		0	0	1	1	0		120
	21R14	0	0	0		0	0	0	0	0		116
	21R21	1	0	0		1	0	0	1	0		113
	21R41	0	0	0		0	0	0	0	0		110
	21R51	2	2	2		1	1	2	3	2		101
	21R61	0	0	0		0	0	0	0	0		113
	21R71	0	0	0		0	0	0	0	0		113
											$LSD_{0.01}$	12
	control	0	0	0		0	0	0	0	0		100

[1] Disease severity ratings according to five classes with: 0: healthy, no symptoms, 1: few small lesions and/or symptoms unclear, 2: slightly infected, 3: moderately infected, 4: heavily infected, or sprout not emerged, -: not determined. 0 and 1 are considered as non-pathogenic under the test conditions.

[2] S.L.: Stem Length of lily as percentage of the control.

[3] R: Red Riding Hood; G: Gander; A: Apeldoorn; BM: Blue Magic; PB: Prof. Blaauw; WE: White Excelsior; PP: Pink Pearl; JB: Jan Bos; E: Enchantment.

[4] c.i.: Isolates with same letters are not significantly different ($P<0.05$) after comparison of treatment means on the underlying scale using a normal approximation. n.i.: not included in the analysis.

[5] c.c.: Cultivars with same letters are not significantly different ($P<0.05$) after comparison of treatment means on the underlying scale using a normal approximation.

Table 5. Average disease severity[1] of four flower bulbs and seedlings of five crucifers and lettuce grown in soil infested with isolates of *Rhizoctonia solani* AG 2-t and AG 2-1 for six weeks (bulbs) and four weeks (seedlings) in the glasshouse at 18 °C. Legend as of Table 4

		leaf						bulb				S.L.[2]	% damping-off					
	crop	tulip				hyacinth		iris				lily	cauliflower	fodder radish	white mustard	oil-seed rape	swede	lettuce
	cv.[3]	R	G	A		PP	JB	BM	PB	WE		E	Oberon	Nemex	Maxi	Jet Neuf	Friese Gele	Petty
	isolate				c.i.						c.i.[4]							
2-t	2tR002	4	4	3	ab	0	0	2	0	1	abc	97	100	85	97	97	69	13
	2tR105	4	4	3	ab	1	2	3	1	3	ef	-	100	100	100	91	100	16
	2tR114	4	4	3	b	1	2	2	1	1	cde	99	100	100	100	97	94	19
	2tR118	4	4	3	ab	-	0	1	0	1	a	94	100	100	97	100	100	47
	2tR123	4	4	3	ab	1	3	2	0	2	cd	-	-	-	-	-	-	-
	2tR124	3	4	3	ab	1	1	2	0	2	abcd	-	100	100	100	97	100	32
	2tR128	4	4	4	b	1	2	1	0	2	bcd	94	100	100	97	94	100	16
	2tR135	4	4	3	ab	1	2	3	0	3	de	88	100	100	92	97	100	10
	2tR138	4	4	3	ab	1	1	1	1	1	abcd	91	100	100	100	77	91	10
	2tR139	4	3	3	a	1	2	1	0	1	ab	92	100	100	100	100	100	22
	2tR144	4	4	3	a	1	2	3	2	3	f	97	100	100	100	100	100	49
	c.c.[5]	a	a	b				a	b	a	$LSD_{0.01}$	9	7	31	8	17	19	35
2-1	21R01	4	4	3		1	1	2	0	1		112	100	-	-	-	-	-
	21R11	1	0	1		1	1	0	0	0		106	100	100	100	100	100	23
	21R12	-	-	-		-	-	-	-	-		-	94	97	94	100	97	7
	21R14	1	0	1		0	0	0	0	1		110	97	100	100	97	100	0
	21R21	1	0	1		0	1	1	0	1		113	100	100	100	94	100	4
	21R41	0	0	1		0	0	0	0	0		109	100	100	94	100	100	0
	21R51	3	3	3		1	1	3	1	2		109	97	100	97	97	100	38
	21R61	0	0	0		0	0	0	0	0		113	100	100	81	100	100	0
	21R71	0	0	0		0	0	1	0	0		110	100	100	87	100	100	3
	21R81	-	-	-		-	-	-	-	-		-	87	54	57	84	85	79
											$LSD_{0.01}$	5	17	36	25	12	8	29
control		0	0	0		0	0	0	0	0		0	6	25	0	0	0	0

Table 6. Average disease severity[1] of four flower bulbs grown in soil infested with isolates of *Rhizoctonia solani* AG 2-2, AG 3, AG 4, AG 5, AG 6, and AG BI for six weeks in the glasshouse at 18 °C

		leaf					bulb			S.L.[2]
		tulip			hyacinth		iris			lily
	c.v.[3]	R	G	A	PP	JB	BM	pB	WE	E
AG	isolate									
2-2	22R01	0	0	0	2	1	2	0	2	104
	22R02	0	0	0	0	0	1	1	1	107
	22R11	4	3	3	1	1	4	2	3	106
3	03R01	0	0	0	0	0	0	0	0	107
	03R03	0	0	0	0	0	0	0	0	96
	03R04	0	0	0	0	0	0	0	0	105
	03R05	0	0	0	0	0	0	0	0	97
4	04R11	4	3	3	1	2	3	4	4	90 **
	04R22	3	3	3	1	2	3	4	4	93 *
	04R10	3	4	3	0	2	3	4	4	99
5	05R01	2	2	1	1	3	0	0	1	103
	05R06	2	3	2	1	3	1	0	2	102
	05R21	1	3	2	1	3	1	0	0	113 **
	05R31	2	3	3	1	3	0	1	1	109 *
6	06R01	0	0	0	0	0	0	0	0	109
BI	BIR01	1	3	2	1	2	2	0	3	102
	control	0	0	0	0	0	0	0	0	0

[1] Disease ratings according to five disease classes with: 0: healthy, no symptoms, 1: few small lesions and/or symptoms unclear, 2: slightly infected, 3: moderately infected, 4: heavily infected, or sprout not emerged, -: not determined. 0 and 1 are considered as non-pathogenic under the tested conditions.

[2] S.L.: Stem Length of lily in percentage of the control. Means significantly different from the control are indicated with **($P<0.01$) and *($P<0.05$).

[3] R: Red Riding Hood; G: Gander; A: Apeldoorn; BM: Blue Magic; PB: Prof. Blaauw; WE: White Excelsior; PP: Pink Pearl; JB: Jan Bos; E: Enchantment.

new AG 2 subgroup distinct from AG 2-1 premature. Our pathogenicity tests showed that all tested AG 2-t and two AG 2-1 isolates (21R01 and 21R51) caused disease in the tested cruciferous and bulbous cultivars (Tables 4 and 5). In addition, isolate 21R06 originating from tulip in Japan was pathogenic to tulip in our experiments (Schneider, unpubl.). The other six Dutch AG 2-1 isolates were pathogenic to cruciferous crops but not to bulbous crops. Naito and Kanematsu (1994) assigned leaf spot isolates from soybean, fusing in high FF with AG 2-1 isolates, to AG 2-3 rather than to AG 2-1 using differences in thiamine requirement and host range as prevailing criteria. Therefore, we suggest designating AG 2 isolates infecting bulbous crops to AG 2-t, in order to distinguish them from other AG 2-1 isolates that are non-pathogenic to bulbous crops. Further research in our institute on characterization of AG 2 isolates using biochemical and molecular methods may provide the means to discriminate unambiguously AG 2-t isolates from AG 2-1 and reveal the (phylo)genetic relationship of AG 2-t and AG 2-1 with other AG 2 isolates.

The observed differences in aggressiveness between AG 2-t isolates (Tables 4 and 5) are in agreement with previous reports on AG 2 subgroups (Doornik, 1981; Kaminski and Verma, 1985; Yitbarek et al., 1987) and other AGs (Carling and Leiner, 1990; Philips, 1991; Carling et al., 1994). In our experiments AG 2-t isolates were in general as aggressive on tulip sprouts at 18 °C as at 9 °C, which is in contrast with Doornik who reported fewer sprouts with severe symptoms at 18 °C. Doornik (unpubl.) noticed considerable differences in symptom expression between years, especially at higher temperatures. A possible explanation for the discrepancy in observations may be explained by differences in susceptibility of bulbs due to storage (duration and other conditions) and treatment to break dormancy. Our experiments were conducted simultaneously at both 9 and 18 °C using the same plant material, inoculum and unsterilised soil mixture.

In our glasshouse tests tulip cv. Apeldoorn was less susceptible to *R. solani* AG 2-t than cvs Gander and Red Riding Hood. Tulip cultivars possessing resistance to *R. solani* have not been reported previously. The only noted differences in cultivar susceptibility are based on farmers experiences (Van Keulen and Van Aartrijk, 1993). For a classification of bulbous crops according to their susceptibility to rhizoctonia bare patch, appropriate experiments are necessary. In such studies the use of more than one isolate may be required, since isolates can differ in aggressiveness. In one isolate/cultivar combination interaction was found, meaning that the cultivar susceptibility depended on the isolates used. Interactions between AG 2-t isolates and tulip cvs under semi-field conditions and in the glasshouse are to be described elsewhere (Schneider, unpub.).

In commercial bulb production fields, cruciferous crops like fodder radish, white mustard, and oil-seed rape are often used as a rotation crop of which the effects on rhizoctonia bare patch are unknown. Pathogenicity tests (Table 5) demonstrated that these crops were susceptible to AG 2-t. Therefore, they must be considered as potential hosts in the field and hence as potential inoculum sources, especially when these crops are used as a green manure. In addition to cruciferous crops, cruciferous weeds may provide alternative hosts in the field. In certain parts of the Netherlands, flower bulbs are grown in rotation with potato, wheat or maize. AG 2-t did not cause damping-off in seedlings of these crops at 18 °C. This finding does not imply, however, that AG 2-t is suppressed or cannot survive in the rhizosphere of these crops under commercial cropping conditions. In fact, AG 2-t isolates could readily be re-isolated from some symptomless seedlings in our glasshouse experiments. Our data suggested that sugarbeets are a potential host for AG 2-t in the field, perhaps depending on the cultivar. The influence of rotation crops, both hosts and non-hosts, on the survival of AG 2-t requires further study in field experiments.

In our glasshouse experiments performed at 18 °C isolates of AG 2-2, AG 4, AG 5, and AG BI, in addition to isolates of AG 2-t, were found to be pathogenic to tulip. Isolate 22R11 caused disease tulip and iris in our glasshouse experiments. *R. solani* AG 2-2IIIB has been reported as a pathogen of gladiolus (Takano and Fujii, 1972) and of sugarbeet (Watanabe and Matsuda, 1966). Because of stromatinia dry rot, gladiolus is grown on the same field once every 20–25 years. Since AG 2-2IIIB causes disease in both gladiolus and sugarbeet, it is evident that cropping gladiolus in short rotation with sugarbeet holds a risk for both crops. AG 3, a well known pathogen of potato, was non-pathogenic to bulbous crops in our experiments. Previously, AG 4 was demonstrated to be pathogenic to bulbous crops (Doornik, 1981). The isolates she used in her studies were obtained from glasshouse grown lettuce and iris, which is in line with the plurivorous nature of this anastomosis group. AG 5 has frequently been isolated from bulbous crops (Loerakker and Van Dreven, 1985; Boerema and Hamers, 1988) and is

generally considered to be a weak pathogen of a great variety of hosts (Sneh et al., 1991). However, O'Sullivan and Kavanagh (1991) demonstrated the pathogenicity of AG 5 isolates to sugarbeet seedlings in glasshouse experiments. Their AG 5 isolates were isolated from diseased sugarbeet seedlings in the field. *R. solani* AG 6 and AG BI are generally considered to be non-pathogenic (Sneh et al., 1991). Recently, AGs 5 and 6 were found strongly pathogenic on apple roots and involved in the 'apple replant disease' (Mazzola, 1996). To our knowledge, this is the first report which describes AG BI as a pathogen (Table 6). Whether AG 6 and AG BI occur in field grown flower bulbs is unknown. It is concluded that, in addition to bare patch causing AG 2-t isolates, isolates representing AG 2-2, AG 4 and AG 5 can cause disease to flower bulbs in glasshouse experiments. Since these isolates are pathogenic at higher temperatures and not at lower temperatures, it is anticipated that these AGs cause bulb rot rather than bare patch. Once the complex of AGs and their impact on bulb production is unravelled, optimal crop rotation schedules, and other methods of environmentally friendly control can be developed.

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