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Investigation of soilborne mosaic virus diseases transmitted by *Polymyxa graminis* in cereal production areas of the Anatolian part of Turkey

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Abstract *Polymyxa graminis* is the vector of several important viruses, including *Soilborne cereal mosaic virus*, *Wheat spindle streak mosaic virus*, *Barley yellow mosaic virus* and *Barley mild mosaic virus*, of winter cereals worldwide. Surveys were carried out to detect these viruses and their vector *P. graminis* in 300 soil samples from the main wheat and barley production areas of the Anatolian part of Turkey collected in May 2002, June 2004 and May 2005. For these surveys, various susceptible wheat and barley cultivars were pot grown in the collected soil samples in a greenhouse and then analysed using ELISA and RT-PCR to detect the presence of different virus species. In addition, a combination of light microscopy following roots staining with acid fuchsin and PCR was used for detection of *P. graminis*. All soil samples analysed were found to be free of these soilborne viruses and their vector.

Keywords BaYMV · BaMMV · Plasmodiophorid · SBCMV · WSSMV · Wheat

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Introduction

Wheat and barley are major crops for human nutrition in most of the countries in the world including Turkey. Wheat is the number one crop in Turkey with the total production area of 7.58 million hectares and a total production quantity of 17.78 million metric tonnes. Barley is the second major crop in Turkey with the total production area of 2.73 million hectares and a total production quantity of 5.92 million metric tonnes (The Food and Agriculture Organization of the United Nations Statistical Database 2008).

Polymyxa graminis (Ledingham 1939), is an obligate root-infecting organism that was originally described from wheat and which has since been found in roots of many cereal and grass species in many parts of the world (Ward et al. 2004). *P. graminis* is a member of the Plasmodiophorales (Karling 1968).

Plasmodiophorids were traditionally considered to be Fungi however an analysis of their ribosomal DNA sequences has revealed that these organisms should probably be classified within the Protists (Ward et al. 2004). Recent studies of the actin and ubiquitin genes confirmed this classification (Archibald and Keeling 2004). *P. graminis* causes no discernable damage to the various host plant species. But it is the vector of several important viruses, including the bymoviruses *Barley yellow mosaic virus* (BaYMV), *Barley mild mosaic virus* (BaMMV) and *Wheat spindle streak mosaic virus* (WSSMV), and the furoviruses *Soilborne wheat mosaic virus* (SBWMV) and *Soilborne cereal mosaic virus* (SBCMV) (Kanyuka et al. 2003). All these soilborne viruses are known to cause yield-limiting mosaic diseases of winter cereals worldwide (Hunger et al. 1989). The viruses are thought to be carried within the *Polymyxa* resting spores (Driskel et al. 2004), and swimming zoospores released upon germination of resting spores have been shown to carry the viruses (Adams 2002). Different species of soilborne viruses cause similar symptoms on susceptible plants, i.e. chlorotic or spindle-shaped streaks and light green or yellow mosaic on infected leaves (Clover and Henry 1999; Barbosa et al. 2001). Even though symptom severity differs from one year to another, these diseases can cause substantial yield losses, e.g. 30–50% on *Triticum aestivum* or 40–80% on *T. durum* (Gilet 1996) and 20–45% on barley (Adams 2002). *P. graminis* resting spores are able to persist in the soil for more than 10 years while remaining viruliferous (Usugi 1988). Consequently, crop rotation is not a satisfactory means to limiting the diseases, nor is chemical control, which is inefficient, expensive and ecologically damaging (Kusuba et al. 1971; Adams et al. 1993). Therefore, control of soilborne mosaic diseases is primarily based on the use of resistant varieties (Kanyuka et al. 2003).

Up to now, soilborne virus diseases of wheat and barley have generally been considered not to be a serious problem in Turkey. To date, there are only a few studies of viruses infecting cereals which are transmitted by *P. graminis* in Turkey. These studies used Enzyme-linked immunosorbent assay (ELISA) tests to show that cereal crops were infected with SBWMV (Köse and Ertunc 1999; Ilbagi and Citir 2004). Also, Kurcman (1981) detected SBWMV infected wheat in the agricultural field in Eskisehir province in the Central Anatolia region of Turkey and investigated the effects of wheat varieties and sowing dates on the severity of infection and crop yield. This early work has been continued by Bolat et al. (1994) who introduced new wheat varieties to the affected areas. More recently Köklü (2004) identified that 13.4% of winter barley samples from Tekirdag province in Thrace, the north-western part of Turkey were infected with BaYMV. Despite this information, there is currently no or only limited data on the presence and distribution of SBWMV, WSSMV, BaMMV and BYMV and their vector *P. graminis* in the major cereal growing areas of Turkey. Therefore, in this study a wide range of soil samples from the Anatolian part of Turkey where cereal production occupies >75% of agricultural land were collected and then analysed for their possible infestation with *P. graminis* and the soilborne viruses it transmits.

Materials and methods

Soil material

During May 2002, June 2004 and May 2005, yellow mosaic and stunting symptoms were observed on wheat and barley plants in fields in Adana, Afyon, Amasya, Ankara, Aksaray, Aydın, Balikesir, Bolu, Cankiri, Corum, Denizli, Diyarbakir, Erzincan, Erzurum, Eskisehir, Igdir, Izmir, Kastomonu, Kayseri, Kirikkale, Konya, Manisa, Mersin, Nevsehir, Samsun, Sivas, Tokat, Urfa, Usak and Yozgat provinces in the Anatolian part of Turkey. A total number of 300 soil samples from major cereal growing fields, having virus like symptoms on plants, were collected randomly by taking 20 surface samples (0–20 cm depth) in each field (Fig. 1 and Table 1). These soil samples were used in bioassays under greenhouse conditions for detection of SBCMV, WSSMV, BaMMV, BaYMV and their vector *P. graminis*. Soil samples were air-dried and then stored at room temperature prior to the inoculation of susceptible 'bait' plants.

Bait plant tests

After all stones and visible roots were removed from the soil samples, each sample was ground using a wooden hammer and passed through a 2 mm aperture sieve. In test 1, seeds of the SBWMV susceptible

Fig. 1 Map of Turkey showing the provinces where the soils sampling was conducted during the 2002, 2004 and 2005 growing seasons. The Anatolian and the Thrace parts of Turkey are shown in black and grey, respectively

wheat cv. Kutluk 94 and barley cv. Tokak 79 (Bolat et al. 1999; Altay and Bolat 2004), were planted into 300 ml plastic pots (10 seeds per pot) containing each of the 300 soil samples mixed with sand (1 part soil : 2 parts sand). Two replicate pots of each soil were placed in a controlled environment room at 19°C day and 18°C night with a 16 h photoperiod. The plants were watered generously every other day with a nutrient solution (Adams et al. 1986). Approximately 4 weeks post inoculation, when the plants had reached growth stage 4 on the Feekes scale (Large 1954), each was trimmed to 5 cm from the soil level to stimulate systemic virus movement (Kanyuka et al. 2004), and harvested 4 weeks later. These plant samples were kept at -20°C until being analysed for the presence of soilborne viruses using ELISA.

In test 2, pre-germinated seeds of wheat cv. Crousty susceptible to SBCMV and WSSMV (S. Berry, Limagrain UK Ltd, personal communication) and barley cv. Maris Otter susceptible to BaYMV and BaMMV (Adams 1991) were planted into plastic pots (5 seeds per pot) containing each of the selected twenty soil samples (Table 2) mixed with sand (1 part soil: 2 parts sand). These soil samples for bait plant inoculation were selected based on the ELISA results obtained in test 1. As a positive control for the inoculation procedure several soils (from the Rothamsted Research collection) infested with *P. graminis* / soil-borne viruses were used. The same soil controls were also used in the concurrent parallel studies, some of which have already been published (Lyons et al. 2009). This test was conducted at two different temperatures in greenhouse compartments: (a) 12°C day / 12°C night (wheat and barley baits), and (b) 20°C day / 16°C night (wheat bait plants only). All bait plants were watered every other day with a nutrient solution (Adams et al. 1986). These conditions were chosen on the basis of data from similar, previous glasshouse-based experiments with SBWMV (Armitage et al. 1990; Kanyuka et al. 2004) and *P. graminis* transmitted bymoviruses of barley (Adams et al. 1986). The bait plants were trimmed to about 5 cm from the soil level at ~ 4-5 weeks post inoculation, and allowed to grow for additional 4 weeks. Then, these plants were harvested and kept at -80°C until tested for the presence of soilborne viruses using ELISA.

Serological assays for detection of soilborne viruses

In test 1, roots and the youngest leaves of the bait wheat plants and roots of the bait barley plants from each pot were taken for the analyses. Leaves and roots of the bait plants from the same pot were combined for sample preparation. Root and leaf extracts of the bait plants were prepared in the presence of 10 volumes of extraction buffer per 1 g fresh weight of the material. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) method was performed according to Arif et al. (1994) using

Table 1 Numbers of soil samples collected in each province of Turkey

Province	Districts	No. of collected samples
Adana	Ceyhan, Yumurtalik	4
Afyon	Sandikli, Dinar, Bayat, Dazkiri	8
Amasya	Centrum, Merzifon, Tasova, Gumushacikoy, Suluova	15
Aksaray	Ortakoy, Gulagac, Guzelyurt	10
Ankara	Elmadag, Beypazari, Golbasi, Polatli, Ayas	10
Aydin	Soke, Kosk, Nazilli	7
Balikesir	İvrindi, Gomec, Burhaniye	6
Bolu	Gerede, Yenicaga	8
Cankiri	Cerkes, Kizilirmak, Atkaracalar, Kursunlu	10
Corum	Alaca, Mecitozu, Osmancik, Iskilip, Lacin, Sungurlu	15
Denizli	Civril, Cardak, Honaz	9
Diyarbakir	Ergani, Silvan, Cinar	15
Erzincan	Centrum	8
Erzurum	Pasinler	2
Eskisehir	Centrum, Alpu, Seyitgazi, Sivrihisar, Mahmudiye	26
Igdir	Tuzluca	1
Izmir	Menderes, Torbali	4
Kastamonu	Centrum, Taskopru	3
Kayseri	Melikgazi, Talas, Hacilar, Yahyali	10
Kirikkale	Delice, Balıseyh, Yahsihan	10
Konya	Centrum, Kulu, Cihanbeyli, Altinekin, Sarayonu, Kadinhani	14
Manisa	Kula	4
Mersin	Centrum, Erdemli, Gulnar	4
Nevsehir	Urgup, Avanos, Gulsehir	8
Samsun	Havza, Alacam, Bafra, Kavak, Vezirkopru	21
Sivas	Centrum, Hafik, Ulas, Yildizeli	18
Tokat	Centrum, Pazar, Turhal, Zile, Erbaa,	15
Urfa	Centrum, Harran, Suruc, Hilvan, Akcakale, Viransehir, Sivelek, Bozova	16
Usak	Centrum, Sivasli	3
Yozgat	Centrum, Yerkoy, Sefaatli	8
Total number of samples =		300

commercial BaMMV, BYMV, SBWMV and WSSMV polyclonal antibodies (Loewe Biochemica GmbH, Sauerlach, Germany) at the manufacturer recommended dilutions. The positive and negative controls provided from Loewe were used in ELISA tests. Absorbance values were read at 405 nm (A_{405nm}) using a Spectra II Microplate Reader (Tecan, Grödig, Austria).

In test 2, a modification of the indirect ELISA method, F(ab')$_2$ ELISA, was used for detection of SBCMV, BaMMV and BaYMV in roots of bait plants, and for re-testing selected root samples, which were classified as putatively positive in test 1. F(ab')$_2$ ELISA utilised in-house produced virus species specific rabbit IgG and F(ab')$_2$ fragments of IgG for SBCMV, BAMMV and BaYMV, and was performed essentially as described in Kanyuka et al. (2004). Root extracts of the bait plants were prepared using Juice Press (Erich Pollähne GmbH, Wennigsen, Germany) in the presence of 10 volumes of extraction buffer per 1 g fresh weight of root material. Absorbances were measured at 405 nm (A_{405nm}) using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

Table 2 Absorbance values (A_{405nm}) in DAS-ELISA tests for detection of SBCMV, WSSMV, BaMMV and BaYMV in leaves and / or roots of bait wheat or barley plants grown in selected soil samples under controlled environment conditions in test 1

Sample No. material	Provinces	Tested plant	ELISA A_{405} values							
			SBCMV		WSSMV		BaMMV		BaYMV	
			2 h[a]	16 h[b]	2 h	16 h	2 h	16 h	2 h	16 h
74	Eskisehir	wheat root	0.313	1.804	–	–	–	–	–	–
91	Eskisehir	wheat root	0.265	1.443	–	–	–	–	–	–
100	Urfa	wheat root	0.201	0.698	–	–	–	–	–	–
101	Urfa	wheat root	0.186	0.695	–	–	–	–	–	–
		barley root	0.130	0.708	–	–	–	–	–	–
112	Diyarbakir	wheat root	0.222	0.740	–	–	–	–	–	–
		barley root	–	–	–	–	–	–	0.150	0.402
149	Yozgat	wheat root	0.202	0.661	–	–	0.095	0.293	0.155	0.468
		barley root	–	–	–	–	–	–	0.166	0.421
157	Ceyhan	wheat root	0.201	0.642	–	–	–	–	–	–
165	Corum	wheat root	–	–	–	–	–	–	0.312	1.052
204	Afyon	wheat leaf	0.187	0.780	–	–	–	–	–	–
223	Tokat	wheat leaf	0.192	0.708	–	–	–	–	–	–
228	Tokat	wheat leaf	0.198	0.821	–	–	–	–	–	–
233	Erzincan	wheat root	–	–	–	–	–	0.309	–	–
236	Erzincan	wheat leaf	0.200	0.836	–	–	–	–	–	–
		barley root	–	–	–	–	–	–	0.173	0.695
253	Amasya	wheat leaf	0.197	0.843	–	–	–	–	–	–
256	Amasya	wheat leaf	0.188	0.733	–	–	–	–	–	–
259	Corum	wheat leaf	0.204	0.844	–	–	–	–	–	–
312	Corum	wheat root	0.204	0.724	–	–	–	–	–	–
320	Corum	wheat root	0.205	0.725	–	–	–	–	–	–
366	Samsun	wheat root	–	–	–	–	–	–	0.158	0.483
373	Samsun	wheat root	0.174	0.536	–	–	–	–		0.604
Negative control[c]			0.111	0.351	0.097	0.157	0.090	0.124	0.101	0.210
Positive control[d]			0.826	2.037	0.187	0.238	0.126	0.438	0.160	0.477

[a] absorbance values at 2 h substrate incubation

[b] absorbance values at 16 h substrate incubation

[c,d] negative and positive controls for each virus species were provided by the manufacturer (Loewe, Sauerlach, Germany) and included in the ELISA detection Kits; "–=ELISA" values at least two times lower than healthy check

Samples were considered positive when the ELISA absorbance (A_{405nm}) values exceeded the mean of the healthy controls by at least a factor of two.

Microscopy analysis of *Polymyxa graminis*

Root subsamples taken from bait wheat and barley plants were washed to remove soil debris and stained with 0.1% acid fuchsin in lactophenol (Ward et al. 2005). The stained root samples were evaluated for the presence of *P. graminis* thalli or plasmodia, zoosporangia, and cystosori under a light microscope.

Total RNA isolation from bait plants

Total RNA was extracted from frozen leaves and roots samples of the bait wheat grown in soil sample Nos. 74, 86, 100, 101, 112, 149, 165, 223 and 230 and the bait

barley grown in soil sample Nos. 6, 75, 91, 106, 107 and 500, which had previously tested positive for one or more virus species in DAS-ELISA and / or F(ab')$_2$ ELISA. Total RNA was extracted from 100 mg of frozen tissues as described previously (Kanyuka et al. 2004).

DNA extraction from bait plants

The whole root system from each individual bait wheat (grown in soil sample Nos. 74 and 91) and barley plants (grown in soil sample Nos. 6, 75, 89, 134 and 149) was freeze dried and ground with a plastic rod in a 1.5 ml Eppendorf tubes. DNA was extracted from the ground roots using the method of Fraaije et al. (1999).

RT-PCR for detection of soilborne viruses

Total RNA extracts from plant samples, which were classified as putatively positive for soilborne virus(es) in ELISA tests (Table 2), were subjected to a one-step RT-PCR analyses using a DNA Engine PTC-200 Thermal Cycler (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), as follows. A 3–5 μg aliquot of total RNA was added to each reaction mixture containing 400 nM forward and reverse virus species specific primers listed in Table 3, 1× ThermoScript Reaction Mix, *Taq* enzyme mix at 2 U/μl (Platinum Quantitative RT-PCR Thermoscript One-Step System; Invitrogen Ltd, Paisley, UK) and RNaseOut at 0.8 U/μl (Invitrogen Ltd, Paisley, UK). First-strand cDNA

Table 3 Primers used in PCR assays for detection of soilborne viruses and their vector *Polymyxa graminis*

Primer name	Primer sequence (5' to 3')	Target	Primer position within the reference sequence (nt)	Reference sequence	Length of expected fragment (bp)	Previously published
SBWMV-UNIF	AACGGTGTTAGTAARYTRGGKGA	SBCMV RNA2	466–488	AJ298069	338	Clover et al. (2001)
SBWMV-UNIR	AAAGAGTCTTGCGTGTARCAYTC		781–803			
RESK-58	GATTGCATACTCAGGTGGAGAG	BaMMV RNA1	3,121–3,142	AJ242725	939	Kanyuka et al. (unpublished)
MMVPg	CTGTATGTGCGCAGGAGCTAT		4,040–4,060			
M3	ACAGAGCACGAGGAGGAA	BaMMV RNA1	6,038–6,055	AJ242725	898	Peerenboom et al. (1996)
M4	GCATGAGAGATCTACCGG		6,919–6,936			
RESK-36	TCGCTGAAGTTATGAAAATGGA	BaYMV RNA1	3,532–3,553	AJ515484	888	Kanyuka et al. (unpublished)
RESK-38	TTGAGTGCGTCGGTTGTGGT		4,401–4,420			
4800EP	GGCTGCAAGCAGCTGATCCTCTCACTG	BaYMV RNA1	6,472–6,498	AJ515484	1,018	Peerenboom et al. (1996)
4799EP	GCCAAAGGCATATGGATCATAGAACCG		7,464–7,490			
RESK-99	ACCCTCTGGAGAACGCCTGGACCT	Barley *eIF4E* mRNA	223–246	AJ699059	620	Kanyuka et al. (2005)
RESK-102	ACAGCATCCACCCGCTACAAGCTA		819–842			
eIF4e_conv. F	GGACCTTCTGGTTCGACAAC	Wheat *eIF4E* mRNA	137–156	Z12616	276	Lyons et al. (unpublished)
eIF4e_conv. R	CCAGCAAAGTATGCAACCAA		394–412			
PgF1	AACATGTGGATTGTGGGCTATGTG	*P. graminis* ribotype I	396–419	Y12824	292	Ward et al. (2004)
PgR1	AACTCCCATTCTCCACAACGCAA		666–688			
PgF2	ATGTGGATCGTCTCTGTTGCTGGA	*P. graminis* ribotype II	415–438	AJ841287	430	
PgR2	CCTCATCTGAGATCTTGCCAAGT		822–844			

Table 4 Absorbance values (A_{405nm}) in DAS-ELISA tests for detection of WSSMV and F(ab')$_2$ ELISA tests for detection of SBCMV, BaMMV and BaYMV in bait wheat or barley plants grown in selected soil samples under controlled environment conditions in test 2

Sample No	District / Province	ELISA A_{405} values									
		SBCMV	WSSMV			BaYMV			BaMMV		
		20°C / 16°C	20°C / 16°C	12°C / 12°C		20°C / 16°C	12°C / 12°C		20°C / 16°C	12°C / 12°C	
		Wheat root	Wheat root	Wheat leaf	Barley leaf	Wheat root	Wheat leaf	Barley leaf	Wheat root	Wheat leaf	Barley leaf
74	Alpu / Eskisehir	0.026	0.230	0.207	0.208	-0.053	0.010	0.017	0.017	-0.044	-0.023
91	Alpu / Eskisehir	0.010	0.151	0.190	0.178	-0.026	0.088	0.016	0.016	-0.018	0.004
100	Harran / Urfa	0.084	0.173	0.113	0.262	-0.030	0.005	-0.024	-0.024	-0.030	-0.011
101	Suruc / Urfa	0.021	0.149	0.253	0.159	-0.054	0.042	0.005	0.005	0.001	-0.010
112	Karabas / Diyarbakir	0.121	0.227	0.105	0.118	-0.029	0.027	-0.031	-0.031	-0.033	-0.040
149	Yerkoy / Yozgat	0.032	0.170	0.154	0.201	-0.048	-0.010	0.013	0.013	0.026	-0.006
157	Centrum / Ceyhan	-0.004	0.171	0.184	0.218	-0.048	0.147	-0.018	-0.018	-0.015	-0.007
165	Mecitozu / Corum	0.011	0.197	0.191	0.157	-0.015	0.069	-0.030	-0.030	0.006	-0.013
204	Sandikli / Afyon	0.070	0.169	0.071	0.155	-0.043	0.046	0.032	0.032	-0.038	0.000
223	Pazar / Tokat	0.038	0.171	0.181	0.080	-0.021	0.049	-0.010	-0.010	-0.039	-0.016
228	Zile / Tokat	0.039	0.167	0.144	0.204	-0.043	0.086	0.000	0.000	0.007	0.004
233	Sarayovasi / Kastamonu	n.t	n.t	0.162	0.156	n.t.	0.043	-0.012	n.t.	0.002	-0.010
236	Bayirbag / Erzincan	0.028	0.202	0.063	0.096	-0.045	0.074	-0.007	-0.007	-0.002	-0.006
253	Merzifon / Amasya	0.011	0.149	0.102	0.111	-0.056	0.088	0.019	0.019	-0.003	-0.051
256	Gümüshacikoy / Amasya	0.055	0.167	0.117	0.088	-0.046	-0.037	0.001	0.001	-0.006	0.014
259	Lacin / Corum	0.048	0.182	0.104	0.086	-0.045	-0.041	0.007	0.007	-0.001	-0.022
312	Osmancik / Corum	0.017	0.156	0.177	0.141	-0.037	0.006	0.010	0.010	-0.044	-0.022
320	Iskilip / Corum	0.017	0.144	0.111	0.172	-0.059	-0.033	0.028	0.028	-0.018	0.026
366	Bafra / Samsun	0.018	0.221	0.148	0.098	-0.043	0.033	0.012	0.012	-0.042	-0.006
373	Vezirkopru / Samsun	0.062	0.186	0.131	0.037	0.026	0.029	0.045	0.045	-0.019	-0.038
- C[a]		0.040	0.170	0.045	0.132	-0.021	0.058	-0.043	-0.043	-0.009	-0.007
+ C[b]		over	3.487	3.487	3.487	0.021	0.160	0.266	0.260	0.265	0.265

[a,b] negative and positive controls for WSSMV, BaYMV and BaMMV were provided by the manufacturer (Loewe, Sauerlach, Germany) and included in the ELISA detection Kits; for SBCMV were used SBCMV-containing and virus-free wheat leaves

synthesis was carried out at 50°C for 30 min and was immediately followed by PCR. Cycling conditions were: 5 min at 95°C; then, 40 cycles, including denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 1 min at 72°C; followed by 10 min at 72°C. In addition, as a quality control measure, each total RNA sample was subjected to a one-step RT-PCR analysis using primers (Table 3) targeting mRNA of an endogenous housekeeping plant gene, *eIF4E* (data not shown). The RT-PCR products were analysed by electrophoresis in 1% agarose gels, followed by staining with ethidium bromide and visualisation under UV light using the GeneGenius Imaging System (Syngene, Cambridge, UK).

PCR for detection of *Polymyxa graminis* in roots of bait plants

To determine whether *P. graminis* was present in roots of bait barley and wheat plants, 100 ng DNA aliquots were used as templates for amplification of *P. graminis* ribotype I and ribotype II using primers Pg.F1, Pg.R1 and Pg.F2, Pg.R2 (Table 3) essentially as described previously (Ward et al. 2005). The PCR products were resolved electrophoretically in 1.2% agarose gels and then visualised under UV following staining with ethidium bromide.

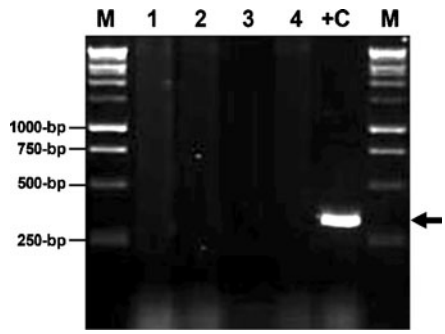

Fig. 2 An example of RT-PCR analysis for detection of *Soilborne cereal mosaic virus*. M: 1 kb DNA Ladder (Promega). The arrow indicates the SBCMV-specific 338-bp DNA fragment only detectable in the positive control sample lane. Lanes 1 and 2: leaf and root RNA, respectively, from a bait wheat plant grown in the soil sample No. 74 from Alpu district in Eskisehir province; lanes 3 and 4: leaf and root RNA, respectively, from a bait wheat plant grown in the soil sample No. 223 from Pazar district in Tokat province; + C: positive control (SBCMV-containing wheat leaves)

Results

To estimate the infectivity of *P. graminis* in sampled soils, a baiting technique was used. In this technique wheat and barley genotypes recognised as susceptible to *P. graminis* as well as to one or more soilborne viruses were pot grown in a greenhouse in soils sampled from agricultural fields in the Anatolian part of Turkey. Eight-nine weeks post inoculation these bait plants were assessed for the presence of *P. graminis* in their roots using microscopy and PCR techniques, and for the presence of associated viruses, SBWMV, WSSMV, BaYMV and BaMMV, in both young leaves and roots using a combination of serological (i.e. ELISA) and molecular biology techniques (i.e. RT-PCR).

Test 1

In test 1, at any time during the growing period under controlled temperature conditions at 19°C day / 18°C night, none of the bait plants displayed typical virus symptoms. A total of 300 root and leaf samples from bait wheat and barley plants were tested for the presence of SBCMV, WSSMV, BaMMV and BaYMV by DAS-ELISA. Colour development in the DAS-ELISA tests using commercial virus-specific antibodies with these samples as well as with the corresponding manufacturer-provided positive con-

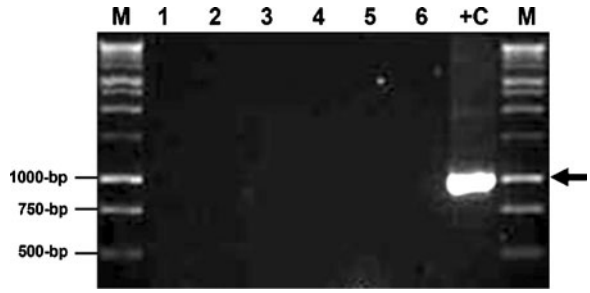

Fig. 3 An example of RT-PCR analysis for detection of *Barley yellow mosaic virus*. M: 1 kb DNA Ladder (Promega). The arrow indicates the BaYMV-specific 1,018-bp DNA fragment only detectable in the positive control sample lane. Lane 1–3: root RNA from barley bait plants grown in the soil samples No. 75 and No. 91 (both from Alpu district in Eskisehir province), No. 106 and No. 107 (both from Suruc district in Urfa province); lanes 5-6: root RNA from the wheat bait plants grown in the soil samples No. 165 (from Mecitozu district in Corum province) and No. 230 (from Erbaa district in Tokat province); + C: positive control (BaYMV-containing barley leaves)

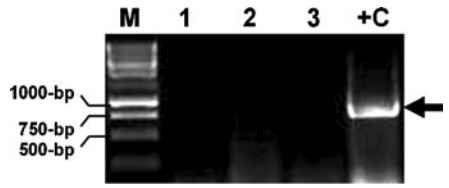

Fig. 4 An example of RT-PCR assay for detection of *Barley mild mosaic virus*. M: 1 kb DNA Ladder (Promega). The arrow indicates the BaMMV-specific 939-bp DNA fragment only detectable in the positive control sample lane. Lanes 1–3: root RNA from bait barley plants grown in the soil samples No. 100 (from Harran district in Urfa province), No. 106 and No. 107 (from Suruc and Hilvan district in Urfa province); + C: positive control (BaMMV-containing barley leaves)

trols for WSSMV, BaMMV and BaYMV was very slow in this study. For this reason, the DAS-ELISA test microtitre plates were read following 2 h substrate incubation at room temperature and then again following overnight substrate incubation at 4°C. Even after an overnight incubation relatively low A_{405} values were obtained for most of the samples, although some samples had reasonably high A_{405} values (Table 2 and data not shown) and were scored as putatively positive for different soilborne viruses. To verify this classification the roots of these putatively positive bait plants were re-examined for the presence of SBCMV, BaMMV and BaYMV using an indirect $F(ab')_2$ ELISA in test 2 described below. In these latter assays some of the re-tested samples had relatively high A_{405} values even after 2 h of substrate incubation (Table 4 and data not shown). In addition, RT-PCR assays were carried out on total RNA extracted from (a) root and leaf samples from the wheat and barley bait plants grown in soils collected from Amasya (Merzifon district), Urfa (Suruc and Hilvan district), Eskisehir (Alpu and Mahmudiye district), Yozgat (Yerkoy district), Tokat (Pazar district) and Samsun (Bafra district) provinces for detection of SBCMV (Fig. 2), and (b) root and leaf samples from the barley bait plants grown in soils collected from Urfa (Suruc and Hilvan district), Eskisehir (Alpu and Mahmudiye district), Corum (Mecitozu district) and Tokat (Erbaa district) provinces for detection of BaYMV (Fig. 3) and BaMMV (Fig. 4). All samples tested negative for soilborne viruses in these RT-PCR based detection assays.

Roots of 300 barley and 300 wheat bait plants grown for 8 weeks in 1: 2 test soil: sand mixtures were also inspected for the presence of *P. graminis* using the light microscopy technique. *P. graminis* was not detected in roots of any of the bait plants. To support the data obtained in these microscopy analyses DNA extracted from the entire root system of the wheat bait plants grown in the soils from Eskisehir province and from roots of the barley bait plants grown in the soils from Amasya, Eskisehir, Tokat and Yozgat provinces were subjected to the sensitive PCR assays for detection of *P. graminis* ribotype I and ribotype II (for sequences of ribotype-specific primers see Table 3). Again, none of the tested samples was positive for *P. graminis* in these assays (Fig. 5).

Test 2

In test 2, wheat and barley baits were grown at two temperature regimes in twenty selected soil samples putatively infested with one or more soilborne viruses

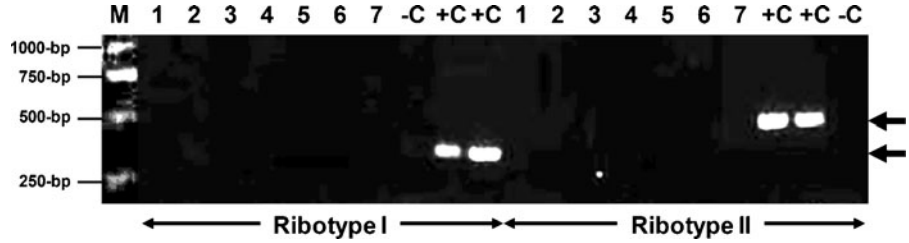

Fig. 5 PCR detection of *Polymyxa graminis*. Expected DNA fragment sizes for *P. graminis* ribotype I and ribotype II are 292-bp and 430-bp, respectively. These are indicated by arrows. M: 1 kb DNA Ladder (Promega). Lanes 1-2: DNA from roots of bait wheat plants grown in the soil samples No. 74 and No. 91 (both from Alpu district in Eskisehir province); lanes 3–7: DNA from roots of bait barley plants grown in the soil samples No. 89 (from Centrum district in Eskisehir province), No. 75 (from Alpu district in Eskisehir province), No. 6 (from Merzifon district in Amasya province), No. 134 (from Centrum district in Tokat province), and No. 149 (from Yerkoy district in Yozgat province). +C: positive control (*P. graminis*-containing wheat roots), –C: negative control (water)

(Table 2). In earlier growth chamber studies, Slykhuis and Barr (1978) determined the optimum temperature range for development of *P. graminis* in wheat roots to be 15° to 22°C, the optimum temperature for transmission of WSSMV to be 15°C, and the optimum temperature for symptom development to be 10°C. In the current study, no virus symptoms were seen on the bait plants grown at constant 12°C or 20°C day / 16°C night temperature regimes. However, yellow striping and yellowing were occasionally seen on the leaves of wheat plants grown at 12°C. Some bait plants also appeared slightly stunted. These symptoms, though, were most probably caused by nutrient imbalance or other abiotic factors, because all bait plants were tested negative for soilborne viruses in both DAS-ELISA and $F(ab')_2$ ELISA tests (Table 4).

Discussion

In this study, the presence of *Polymyxa graminis* and the soilborne cereal viruses it transmits in agricultural fields of thirty provinces in the Anatolian part of Turkey was investigated. In total, 300 soil samples were collected and used in greenhouse pot tests for growing various wheat and barley cultivars (i.e. so called 'bait plants') recognised as susceptible to *P. graminis* and one or more soilborne virus species. Various microscopy, serological and molecular biology pathogen detection tests failed to detect soilborne viruses and their vector in 8-9 week old bait plants.

In a recent survey of wheat samples from agricultural fields in Belgium for the presence of soilborne viruses none of the samples, except for the wheat cv. Centanaire collected in the field near Flavion, were scored as positive for WSSMV, SBCMV or SBWMV in ELISA tests. Interestingly, these viruses were detected in many of these samples using more sensitive RT-PCR assays (Vaianopoulos et al. 2006). In the current study, the RT-PCR virus detection assays were also performed on those samples that were scored as putatively positive in ELISA tests. However, all samples tested negative for SBCMV, BaYMV and BaMMV in these RT-PCR assays (Figs. 2, 3 and 4). It was not possible in this study, which took place in the UK laboratory during 2005, to analyse field-sampled plants displaying virus-like symptoms from Anatolia and additionally confirm the results of the greenhouse transmission experiments due to quarantine regulations. However, more recently several field-grown wheat plants displaying virus-like symptoms sampled from Eskisehir (the same fields as soils used in this study) and Samsun provinces have been tested at Rothamsted Research, UK and Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany. The results of these tests indicated that all samples were free of known wheat-infecting soilborne viruses (RL Lyons, W Huth and ND Kutluk Yilmaz, unpublished).

In this study the roots of 300 barley and 300 wheat bait plants stained with acid fuchsin were also examined for the presence of *P. graminis* under a light microscope. However, *P. graminis* was not detected in these samples (data not shown). The temperate isolates of *P. graminis* are known to colonise barley roots over a wide temperature range although the temperature interval of 17–20°C has been shown to be the most permissive for this microorganism (Adams and Swaby 1988). In this narrow temperature interval typically only ~ 2 weeks is required from plant inoculation with *P. graminis* resting spores to *in planta* production of secondary zoospores. *P. graminis* infection foci are often distributed quite erratically in the root system making the light microscopy aided assessment of infection generally very difficult and perhaps even inaccurate. Consequently, more sensitive and accurate PCR detection methods for *P. graminis* have been developed (Ward et al. 1994; Ward and Adams 1998). These PCR assays target either nuclear 18 S or 5.8 S or internal transcribed spacer regions ITS1 and ITS2 of the nuclear ribosomal DNA (rDNA) (Legreve et al. 2002; Ward et al. 1994; Ward and Adams 1998). Due to sequence differences in the genome regions mentioned above, the two morphologically similar *Polymyxa* species, *P. graminis* and *P. betae*, as well as several subgroups (ribotypes) within the *P. graminis* species can be clearly distinguished in a PCR assay. These distinct rDNA sequence types seem to be related to the host range, temperature requirements and geographical origin. In the temperate geographical zone, ribotypes I and II of *P. graminis* are known to predominate. Optimal growth of these temperate isolates mainly known to infect wheat and barley is achieved at 15–20°C (Legreve et al. 2003). However, tropical isolates of *P. graminis* generally known to infect sorghum, pearl millet, maize and rice have a growth temperature optimum of 27–30°C (Legreve et

al. 1998; Legreve et al. 2003). The biological significance of the two temperate ribotypes is not clear but in an earlier study the majority of *P. graminis* isolates obtained from barley were shown to belong to ribotype I, whereas most ribotype II isolates reported to date have been obtained from wheat or other cereals (Ward et al. 2004). Therefore, to confirm the absence of *P. graminis* ribotype I and/or II, DNA extracted from the entire root system of selected bait plants were tested in the current study using a sensitive PCR detection method. In these PCR assays, none of the samples tested positive for *P. graminis* (Fig. 5).

Collectively, the data obtained in this study did not support the conclusion from the earlier work by Köse and Ertunc (1999) that SBWMV and its vector *P. graminis* may be present in Eskisehir province. Similarly, in another independent study of wheat plants collected from the agricultural fields in Eskisehir province and displaying SBWMV-like symptoms no *P. graminis* structures in the roots of these plants have been detected. Moreover, the shape and the size of virus-like particles that had been observed in the leaves of these plants using electron microscopy and suggested that the causal virus may be a new species rather than SBWMV (Makkouk et al. 1994). In our field surveys conducted in 2005 in Alpu, Mahmudiye and Centrum districts of Eskisehir province, the main symptoms detected on the wheat plants were severe stunting, rosetting, and intense greening of the leaves (Fig. 6b and Kutluk Yilmaz, unpublished). However, the appearance and severity of soilborne mosaic symptoms in wheat is known to vary considerably depending on plant genotype, the concentration and aggressiveness of the virus species or virus strain, as well as the environmental conditions i.e. temperature, moisture, etc. (Budge and Henry 2002). Also, some wheat genotypes may show no visible mosaic symptoms despite the presence of moderate-to-high virus titres in both leaves and roots. Moreover, uneven distribution of fertiliser in the field, nutrient imbalance or winter injuries may cause symptoms (e.g. leaf mosaic, stunting) in pathogen-free plants that could be mistaken for soilborne virus disease (Kanyuka et al. 2004). Indeed, SBCMV, WSSMV, BaMMV and BaYMV were not detected using $F(ab')_2$ ELISA, DAS-ELISA or the more sensitive RT-PCR tests in the bait plants grown in 26 soil samples collected from Eskisehir province.

Fig. 6 **a** Healthy plants; **b** Symptoms seen on wheat plants from Alpu district in Eskisehir province; **c** *Aerobasidium* spp. spores detected in stained roots of wheat plants from Alpu district in Eskisehir province by light microscopy

Moreover, *P. graminis* was also undetected in these samples using light microscopy of acid fuchsin stained roots or the ribotype-specific PCR assays. However, in these same samples the spores of *Aerobasidium* spp., which have a similar appearance to *P. graminis* cystosori under the microscope, were often noted (Fig. 6c).

Soilborne pathogens including *P. graminis* are known to be not uniformly distributed within the field. This may account for the biological differences in collected samples from different fields or different parts of the same field in Eskisehir province. Köse and Ertunc (1999) indicated that the plants grown on SBWMV infected soils under natural conditions had a greatly reduced canopy, were severely stunted, and failed to reach the heading stage. However, it is

important to point out that Köse and Ertunc (1999) detected no reaction against SBWMV in the same samples in a serological agar gel double diffusion and sap inoculation tests. Similarly, in our greenhouse pot inoculation experiments, all bait plants grown in Eskisehir province soils (26 samples) appeared soil-borne virus symptoms free, i.e. healthy. It is also important to note that the virus particles, observed using electron microscopy in the wheat field samples from Eskisehir province in studies by Kurcman (1981) and Makkouk et al. (1994), were ~ 640 nm long rods, whereas SBWMV and related SBCMV virus particles are known to have modal lengths ranging from approximately 45 nm to 420 nm (Gumpf 1971; Lyons et al. 2009). Therefore, the virus(es) detected in these early studies was not likely to be SBWMV.

In conclusion, the results from this study suggest that soilborne cereal viruses transmitted by *P. graminis* are not present in the tested 300 soil samples collected from agricultural fields in major cereal production areas of the Anatolian part of Turkey, and that symptoms observed on cereal plants in previous studies by others (Kurcman 1981; Köse and Ertunc 1999; Altay and Bolat 2004; Ilbagi and Citir 2004) are likely to be caused by other as yet unidentified pathogens or abiotic factors. It is possible that average air / soil temperatures and or amount of precipitation in the Anatolian part of Turkey during the cereals crop growing months (*Polymyxa* species are known to favour wet conditions) are suboptimal for *P. graminis* growth / development. It is therefore possible that *P. graminis* is either completely absent in the Anatolian part of Turkey, or very rare and multiplies to detectable levels only in rare years (when environmental conditions are particularly favourable) and / or can be found only in tiny infested patches within agricultural fields. An alternative explanation for the negative results obtained in this study is that *P. graminis* isolates native to the Anatolian part of Turkey may have significantly higher temperature optima than those documented for *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* from temperate regions with cooler climates. The latter hypothesis will be tested in future bait plant tests.

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