Research Article

Received: 2 July 2008 Revised: 1 December 2008 Accepted: 2 December 2008 Published online in Wiley Interscience: 6 April 2009

(www.interscience.wiley.com) DOI 10.1002/ps.1751

Control of *Fusarium verticillioides*, cause of ear rot of maize, by *Pseudomonas fluorescens*

Siddaiah Chandra Nayaka,[a] Arakere C Udaya Shankar,[a] Munagala S Reddy,[b] Siddapura R Niranjana,[a]* Harishchandra S Prakash,[a] Hunthrike S Shetty[a] and Carmen N Mortensen[c]

Abstract

BACKGROUND: Maize is one of the staple food crops grown in India. *Fusarium verticillioides* (Sacc.) Nirenberg is the most important fungal pathogen of maize, associated with diseases such as ear rot and kernel rot. Apart from the disease, it is capable of producing fumonisins, which have elicited considerable attention over the past decade owing to their association with animal disease syndromes. Hence, the present study was conducted to evaluate ecofriendly approaches by using a maize rhizosphere isolate of *Pseudomonas fluorescens* (Trev.) Mig. and its formulation to control ear rot disease and fumonisin accumulation, and also to study the capacity to promote growth and yield of maize. *In vitro* assays were conducted to test the efficacy of *P. fluorescens* as a seed treatment on seed germination, seedling vigour and also the incidence of *F. verticillioides* in different maize cultivars. The field trials included both seed treatment and foliar spray. For all the experiments, *P. fluorescens* was formulated using corn starch, wheat bran and talc powder. In each case there were three different treatments of *P. fluorescens*, a non-treated control and chemical control.

RESULTS: Pure culture and the formulations, in comparison with the control, increased plant growth and vigour as measured by seed germination, seedling vigour, plant height, 1000 seed weight and yield. *P. fluorescens* pure culture used as seed treatment and as spray treatment enhanced the growth parameters and reduced the incidence of *F. verticillioides* and the level of fumonisins to a maximum extent compared with the other treatments.

CONCLUSION: The study demonstrates the potential role of *P. fluorescens* and its formulations in ear rot disease management. The biocontrol potential of this isolate is more suited for fumonisin reduction in maize kernels intended for human and animal feed.

Keywords: maize; *Fusarium verticillioides*; fumonisins; *Pseudomons fluorescens*; control

1 INTRODUCTION

Maize is an important cereal crop grown in India. It is attacked by several fungal diseases, and, among these, ear and kernel rot caused by *Fusarium verticillioides* (Sacc.) Nirenberg is the most devastating, responsible for significant crop yield losses. *Fusarium verticillioides* is capable of producing several mycotoxins in maize, and fumonisins are the most important contaminants. Fumonisins were discovered in 1988 and are produced by *F. verticillioides*, *F. proliferatum* (Matsushima) Nirenberg and several uncommon *Fusaria*.[1,2] Fumonisins have been found to be very common contaminants of corn-based food and feed in the USA, Asia, Europe and South America.[3] They are toxic and are known to cause encephaloamalacia in horses.[4] Exposure to *F. verticillioides*-contaminated maize has been linked to elevated rates of esophageal cancer.[5] The *Fusarium*-infected maize seeds have poor germinability, and the toxin makes the grain unfit for consumption. Studies on the control of fumonisins in major food crops such as maize are still at a relatively early stage. To date, fungicides such as thiram and carbendazim are used as seed treatments to control the disease in India. There has been little success with these fungicides, and the problem of pesticide residue in seeds/grains is becoming increasingly acute, in addition to the fact that they are expensive. Therefore, there is a serious need for alternative control practices of *Fusarial* disease in maize. Among the many practices, biological control with bacterial or fungal isolates may be an alternative approach. Therefore, in the present study an attempt has been made to employ *Pseudomonas fluorescens* (Trev.) Mig., isolated from rhizosphere of maize, and to evaluate its efficacy in reducing the disease caused by *F. verticillioides* and the toxins, thereby increasing the seed quality of maize.

* *Correspondence to: Siddapura R Niranjana, Biocontrol Research Laboratory, Asian Seed Health Centre DOS in Applied Botany, Seed Pathology and Biotechnology, University of Mysore, Manasagangothri, Mysore 570 006, India. E-mail: srn@appbot.uni-mysore.ac.in*

a *Asian Seed Health Centre, Department of Studies in Applied Botany and Biotechnology, University of Mysore, Manasagangotri, Mysore 570 006, Karnataka, India*

b *Department of Entomology and Plant Pathology, 209 Life Sciences Bldg, Auburn University, AL 36849, USA*

c *Danish Seed Health Centre, Department of Plant Biology, University of Copenhagen, Copenhagen, Denmark*

2 MATERIALS AND METHODS

2.1 Source of maize seeds

Maize cultivars, namely Mysore sweet corn (susceptible to *Fusarium* species), Kanchan (moderately resistant) and Pioneer (resistant), were obtained from the All-India Coordinated Maize Research Centre, Nagenahalli, Mandya District, Karnataka, India.

2.2 PCR amplification of *Fusarium verticillioides*

The toxigenic strain of *F. verticillioides* used in the present study was isolated from maize cv. Mysore sweet corn.[6] The identification of toxigenic *F. verticillioides* was done by fumonisin-producing *F. verticillioides* specific primers VERTF-1 5′ GCGGGAATTCAAAGTGGCC 3′ and VERTF-2 5′ GAGGGCGCGAAACGGATCGGG 3′, and the PCR amplification was carried out in 25 μL of the reaction mixture.[7] The fungus was maintained on potato dextrose agar (PDA). Inoculum was prepared from eight-day-old cultures, and suspensions were adjusted to 10^8 conidia mL^{-1} by haemocytometer for further use.

2.3 Isolation, mass multiplication and preparation of *Pseudomonas fluorescens*

The antagonistic strains of *P. fluorescens* were isolated from the native soil, maintained on King's B medium (KBM) and identification confirmed through polymerase chain reaction using flagellin gene specific primer for *P. fluorescens*.[8] *Pseudomonas fluorescens* was used both as pure culture and as a formulation. Cell suspension (100 μL) of *P. fluorescens* was inoculated into sterile 1000 mL Roux bottles containing 125 mL of KBM. The bottles were placed horizontally and incubated at 26 ± 2 °C under a 12 : 12 h light : dark photoperiod. The culture in KBM (48 h old) was centrifuged at 10 000 × *g* for 5 min. The pellets were resuspended in sterile distilled water, washed twice and suspended again in sterile distilled water. The density of the cells in the suspension was adjusted to 10^9 cfu mL^{-1} with the help of a UV-visible spectrophotometer (Hitachi, Japan).[9]

The production of *P. fluorescens* on different solid substrates such as wheat bran and corn starch was carried out as described below.

Substrates of corn starch and wheat bran were collected from local markets of Karnataka. A quantity of 20 g of each substrate was placed in a 250 mL flask containing distilled water. The contents were mixed thoroughly with the help of a glass rod until all the substrate particles were evenly moistened and no clumps were present. Flasks were than inoculated with 1 mL of *P. fluorescens* containing 10^9 cfu mL^{-1}. The contents were shaken well to disperse the inoculum evenly, and the inoculated flasks were then incubated at 26 ± 2 °C under a 12 : 12 h light : dark photoperiod for 14 days. The initial weight before incubation was noted, and changes in the subsequent weights were noted periodically. Samples (1 g) were drawn from each flask and suspended in 9 mL of sterile distilled water containing 0.2 g L^{-1} Tween-20. The cell concentration of *P. fluorescens* was assessed using a spectrophotometer.

2.4 Talcum formulation

Pseudomonas fluorescens formulation (18×10^7 cfu g^{-1}) was prepared by aseptically mixing 100 mL of *P. fluorescens* suspension and 25 g of talcum powder under sterile conditions. Carboxy methyl cellulose (CMC) (2.5 g) was also added to 250 g of formulation and stored in the form of talc packed in polythene bags under ambient conditions until further use. Surface-sterilised maize seeds were mixed with the formulation at 10 g kg^{-1} seed. Seed treated with talc powder amended with CMC served as an untreated control.

2.5 Seed treatment with *Pseudomonas fluorescens*

Bacterisation of the seed was achieved by soaking seeds in *P. fluorescens* 10^9 cfu g^{-1} suspensions (1 g 25 mL^{-1}), prepared as described in Section 2.3, and amended with 2 g L^{-1} sterilised CMC as a sticker. The suspensions were incubated at 26 °C in a rotary shaker for 6 h to facilitate attachment of bacterial cells to the seed coat. Later, the seeds were allowed to dry in an incubator at 30 °C. Seed treated with sterilised distilled water amended with CMC served as an untreated control.

Carbendazim (Bavistin WP; BASF India Ltd), a standard fungicide recommended for maize seed protection in India, was treated at 2 g kg^{-1} of seeds for comparison. Untreated seeds were used as control.

2.6 Screening for *Fusarium verticillioides* incidence

Four hundred seeds of maize cv. Mysore sweet corn, Pioneer and Kanchan were screened to record the percentage incidence of *F. verticillioides* by a standard blotter method.[6]

2.7 Germination and vigour index test

Maize seeds treated with or without *P. fluorescens* as described in Section 2.5 were subjected to a germination test according to the paper towel method.[6] Seedling vigour was analysed and calculated at the end of 7 days incubation.[10]

2.8 Field studies

Field trials were conducted at Nagenahalli Research Station (All-India Coordinated Maize Improvement Programme, Govt of India) located at Srirangapatna, Karnataka, India. Field trials were conducted for three consecutive years during the crop seasons of 2004–2005, 2005–2006 and 2006–2007. In each experiment there were three different kinds of treatment: (a) seed treatment; (b) foliar spray; (c) seed treatment + foliar spray. The experiment also had an untreated control and chemical control.

Pure culture and formulations of *P. fluorescens* were applied as spray treatments at different stages of plant development such as boot leaf stage, anthesis stage, milky stage and physiologically mature stage at a rate of 18×10^7 cfu g^{-1}. Powder formulation (1 g) was dissolved in 100 mL of distilled water, and each ear head was sprayed completely from the tip of the ear head at all stages.[11] The chemical fungicide carbendazim (2 g L^{-1}) was also sprayed, and plants sprayed with distilled water served as control.

There were five replications per treatment. Each replication was a single row of 5 m length and was hand seeded with 100–150 seeds per row. The field was maintained according to recommended growing condition (red loamy soil, irrigated once in 15 days, with thinning done after 21 days.)

2.9 Field emergence test

The field experiment was conducted to determine the effect of *P. fluorescens* suspensions and powdered formulations on the growth of maize. Treatments were the same as described in Section 2.8. *Pseudomonas fluorescens*-treated seeds were hand sown, and there were five replications per treatment. Each replication was a single row of 5 m length, hand seeded, with 100–150 seeds per row. The field was maintained according to the maize growing conditions (red loamy soil, irrigated once in 15 days and spacing done after 21 days). No chemicals or fertilisers were used. At 30 days after seeding, plant height was recorded.

2.10 Ear rot disease incidence

The plants were observed for ear rot disease development and rated for disease when they showed any one of the typical ear rot/kernel rot symptoms such as a powdery or cottony-pink mould growth on the infected kernels. The data were consolidated at 60 days after sowing. Experiments were conducted twice. The disease was rated on a 0–7 scale by following the method of Reid *et al*.[12]

Ear rot incidence was determined by counting the total number of cobs and total number of infected cobs per plot. Data on percentage incidence of ear rot were analysed by analysis of variance, and the least significant difference test[13] was used for evaluating the significance of differences between the control and the treatments.

2.11 Effect on yield

After complete maturity, maize cobs were harvested, dried and threshed separately. The grains thus obtained were weighed separately, and the grain yield in the sprayed plots with different treatments was compared with that of the unsprayed control plots.

2.12 Screening of maize cultivars for resistance to *Fusarium* ear rot and fumonisin production

A toxigenic strain of *F. verticillioides* isolated from maize seed was inoculated into the maize cultivars by injecting a conidial suspension of *F. verticillioides* into the silk channel inside the husk cavity and above the cob. The inoculation was done 4–7 days after silk emergence when there is a peak in expression of susceptibility. Inoculum (2 mL) was injected into the silk channel of each primary ear using a 10 mL hypodermic syringe. Secondary ears were not inoculated as they were not present in all genotypes and they often mature later than primary ears. Disease severity ratings were recorded on a 1–7 scale, where 1 = no infection and 7 = >75% of the kernels visibly mouldy.

2.13 Fumonisin production

Seeds of two resistant (Pioneer and Kanchan) and one susceptible (Mysore sweet corn) maize cultivars were collected from plants, challenge inoculated with toxigenic *F. verticillioides* and treated with pure culture and formulations of *P. fluorescens* as described earlier, and untreated control and carbendazim-treated seeds were tested for total fumonisin level using commercially available quantitative ELISA assay kits (Neogen Corp., Lansing, MI) according to the manufacturer's instructions.

3 RESULTS

3.1 PCR amplification of *Fusarium verticillioides*

PCR assays with *F.verticillioides* specific primer VERTF-1 and VERTF-2 primers yielded a single PCR product at 400 bp. Overall, the PCR assay confirmed that *F. verticillioides* is toxigenic in nature (Fig. 1).

3.2 PCR amplification of *Pseudomonas fluorescens*

PCR assays with PAFP-1 and PAFR-1 primers amplified a single fragment of about 858 bp when DNA from an isolate of *P. fluorescens* was used (Fig. 2).

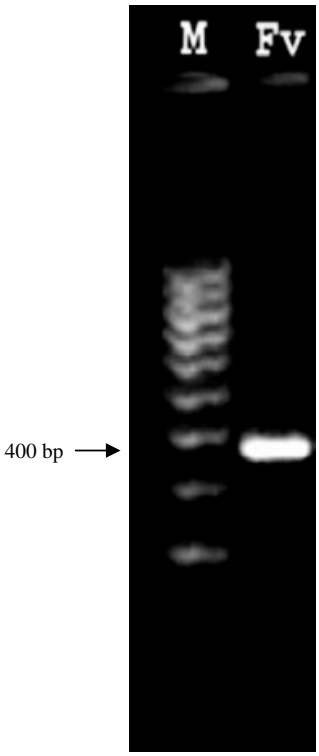

Figure 1. PCR amplification using primers VERTF-1/VERTF-2 and DNA from fumonisin-producing strains of *Fusarium verticillioides*.

Figure 2. PCR amplification using primers PAFP-1/PAFR-1 and DNA from the *Pseudomonas fluorescens* isolate used in the present study.

3.3 Effect of *Pseudomonas fluorescens* on incidence of *Fusarium verticillioides*

The effects of pure culture and different formulations of *P. fluorescens* on the incidence of *F. verticilliodes* in treated seeds compared with control are presented in Table 1. Pure culture of *P. fluorescens* at a rate of 10^9 cfu g^{-1} reduced *F. verticillioides* incidence by 82%. Different powder formulations of *P. fluorescens* also reduced the incidence: talcum powder formulation by 76%, cornstarch formulation by 66% and wheat bran formulation by 59%. Carbendazim also reduced the incidence of *F. verticillioides* by 61% over control.

Table 1. Effect of *Pseudomonas fluorescens* on the incidence of *Fusarium verticillioides*[a,b]

Treatment	Maize cultivars % *Fusarium* incidence		
	Kanchan	Pioneer	sweet corn
***P. fluorescens* (10^9 cfu mL^{-1})**			
Pure culture	5.12a	3.34a	11.77a
Talc formulation 10 g kg^{-1}	7.12b	5.42ab	15.21b
Corn starch formulation 10 g kg^{-1}	9.98c	6.78b	17.12bc
Wheat bran formulation 10 g kg^{-1}	14.19d	9.32c	23.12d
Carbendazim 2 g kg^{-1}	12.01cd	7.37d	17.75c
Control	29.91e	18.77e	46.18e

[a] Mean from three repeat experiments with four replications and 100 seeds per replication in each experiment.
[b] Means within columns sharing the same letters are not significantly different according to Tukey's HSD test at $P = 0.05$.

Table 3. Effect of biological agents on field emergence of maize[a,b]

Treatment	Maize cultivars % Field emergence		
	Kanchan	Pioneer	sweet corn
***P. fluorescens* (10^9 cfu mL^{-1})**			
Pure culture	89d	90d	80d
Talc formulation 10 g kg^{-1}	80c	86c	73c
Corn starch formulation 10 g kg^{-1}	74a	83c	72ab
Wheat bran formulation 10 g kg^{-1}	75b	80c	70b
Carbendizim 2 g kg^{-1}	75b	76b	70b
Control	71a	72a	65a

[a] Values are means from three repeat experiments with four replications and 100 seeds per replication in each experiment.
[b] Means within columns sharing the same letters are not significantly different according to Tukey's HSD test at $P = 0.05$.

3.4 Effect of seed treatment with *Pseudomonas fluorescens* on seed germination and vigour index

In comparison with the untreated control, *P. fluorescens* treatment significantly enhanced both seed germination and seedling vigour in all cultivars of maize (Table 2). Treatment of maize seeds with pure culture of *P. fluorescens* significantly enhanced germination and seedling vigour by 35 and 68% respectively, whereas the talcum powder, corn starch and wheat bran formulations increased germination by 26, 23 and 18% respectively, and seedling vigour by 62, 60 and 58% respectively. Carbendazim treatment increased the germination and vigour by 18 and 60% respectively.

3.5 Effect of seed treatment with *Pseudomonas fluorescens* on field emergence

Maize seeds treated with pure culture of *P. fluorescens* increased field emergence by 22%, and talc, corn starch and wheat bran formulations by 13, 9 and 8% respectively. Carbendazim treatment increased the field emergence by 6% (Table 3).

3.6 Effect of *Pseudomonas fluorescens* on yield

All the treatments increased maize yield in comparison with carbendazim and untreated control (Table 4). Among different treatments, seed + spray treatment with *P. fluorescens* pure culture gave the maximum increase in yield by 33.8% in comparison with control. Powder formulations of talc, corn starch and wheat bran used as seed treatment followed by spray treatment increased the yield by 21%. The chemical fungicide carbendazim increased the yield by 13%.

3.7 Effect of seed treatment with *Pseudomonas fluorescens* on ear rot disease incidence

Under field conditions, significant reduction in ear rot disease was observed in the test rows raised from seeds treated with both pure culture and formulations of *P. fluorescens*, in comparison with the fungicide-treated and untreated control rows (Table 5). The results showed that there was significant reduction in the incidence of ear rot disease in all the cultivars with seed treatment followed by spray treatment using pure culture and powder formulation of *P. fluorescens*. Seed treatment followed by spray treatment of pure culture of *P. fluorescens* reduced the incidence of ear rot disease by 83%. Powder formulations of talcum, cornstarch and wheat bran reduced ear rot by 81, 79 and 77% respectively. The chemical fungicide carbendazim applied as seed treatment followed by spray treatment also reduced the disease incidence by 77% compared with the untreated control.

Table 2. Effect of *Pseudomonas fluorescens* on seed germination and vigour index in maize[a,b]

Treatment	Maize cultivars % Germination			Maize cultivars Vigour index		
	Kanchan	Pioneer	sweet corn	Kanchan	Pioneer	sweet corn
***P. fluorescens* (10^9 cfu mL^{-1})**						
Pure culture	96e	97a	94d	1977d	2198d	1889d
Talc formulation 10 g kg^{-1}	90d	93b	86c	1901d	2119d	1824c
Corn starch formulation 10 g kg^{-1}	87c	89bc	84c	1878c	2087c	1802b
Wheat bran formulation 10 g kg^{-1}	80b	86b	79b	1782b	2002b	1780b
Carbendazim 2 g kg^{-1}	84b	89bc	78b	1799b	2065c	1792b
Control	71a	82a	68a	1278a	1586a	1120a

[a] The values are the mean from three repeat experiments with four replications and 100 seeds per replication in each experiment.
[b] Means designated with the same letter are not significantly different according to Tukey's HSD test at $P = 0.05$.

Table 4. Effect of *Pseudomonas fluorescens* treatments on yield in three different cultivars of maize[a,b]

Treatment	Maize cultivar (q ha^{-1}) Kanchan	Pioneer	Sweet corn
***P. fluorescens* (10^9 cfu mL^{-1})**			
Seed treatment (ST)	88.1a	101.1a	57.4b
Foliar spray (FS)	81.3b	96.5b	47.5a
ST + FS	94.2c	109.2ab	61.7c
Talc formulation 10 g kg^{-1}			
Seed treatment	85.2b	98.6ab	53.5ab
Foliar spray	80.4a	95.4a	52.1a
ST + FS	89.6c	104.2c	55.7c
Corn starch formulation 10 g kg^{-1}			
Seed treatment	82.2b	96.4b	50.6b
Foliar spray	78.3a	94.2a	47.7a
ST + FS	87.3c	101.5c	54.2c
Wheat bran formulation 10 g kg^{-1}			
Seed treatment	79.2b	90.1ab	47.3b
Foliar spray	77.2ab	89.7a	45.7ab
ST + FS	83.5d	96.8d	48.8c
Carbendazim 2 g kg^{-1}	82.0c	95.0c	49.2d
Control	76.4a	89.2a	45.0a

[a] Based on the weight of seeds collected from the central two replicates and converted to quantity per hectare by the procedure of Williams and Singh.[31]
[b] Means within columns sharing the same letters are not significantly different according to Tukey's HSD test at $P = 0.05$.

Table 5. Effect of *Pseudomonas fluorescens* treatment on incidence of ear rot disease in three different cultivars of maize[a,b]

Treatment	Maize cultivar % Disease incidence Kanchan	Pioneer	sweet corn
***P. fluorescens* (10^9 cfu mL^{-1})**			
Seed treatment	6.00b	5.77b	5.92ab
Foliar spray	7.25c	6.27c	6.18c
ST + FS	4.91a	3.27a	5.22a
Talc formulation 10 g kg^{-1}			
Seed treatment	6.37b	5.92b	6.31ab
Foliar spray	7.61c	6.46c	7.38c
ST + FS	5.21a	4.81a	6.01a
Corn starch formulation 10 g kg^{-1}			
Seed treatment	6.87b	6.01b	7.21b
Foliar spray	7.74c	6.71c	7.94c
ST + FS	5.66a	5.18a	6.61a
Wheat bran formulation 10 g kg^{-1}			
Seed treatment	7.07b	7.21b	8.40c
Foliar spray	8.01c	7.41bc	8.85d
ST + FS	6.12ab	5.79d	8.17b
Carbendizim 2 g kg^{-1}	5.59a	6.10a	7.14a
Control	19.1d	13.0d	32.0d

[a] Percentage protection is the mean from three repeat experiments with four replications and 100 seeds per replication in each experiment.
[b] Means designated with the same letter are not significantly different according to Tukey's HSD test at $P = 0.05$.

3.8 Effect of *Pseudomonas fluorescens* seed treatment on fumonisin level in maize samples

The results showed that there was significant reduction in the level of total fumonisins in all the cultivars subjected to seed treatment followed by spray treatment with pure culture and powder formulation of *P. fluorescens*. Seed treatment followed by spray treatment with pure culture of *P. fluorescens* reduced the incidence of fumonisins by 88%, followed by talcum powder formulation (82%), corn starch formulation (75%) and wheat bran formulation (77%). Carbendazim treatment also reduced the fumonisins by 77% over control (Table 6).

4 DISCUSSION

Mycotoxin control measures have been implemented for agricultural commodities entering international trade or located in countries with centralised or large-scale buying and distribution systems. However, in developing countries, where local food consumption or subsistence agriculture is practised by as much as 70% of the population, such measures would be difficult to implement. Because of the stringent mycotoxin control measures being maintained by those countries importing food grains and oilseeds, and because of the need for exporting countries to earn foreign exchange, the best of the commodities are often sold abroad, while the substandard or contaminated commodities are retained for domestic use. After four decades of research in maize, there is still no obvious solution in sight with respect to fungal contamination and elaboration of toxins.[3]

In the present study, three different maize cultivars, Kanchan, Pioneer and Mysore sweet corn, which were considered as moderately resistant, resistant and susceptible, respectively, to *Fusarium* sp., and having different ranges of *F. verticillioides*-infected seeds, were treated with *P. fluorescens* and its formulations and evaluated for *F. verticillioides* incidence, effect on seed germination, seedling vigour, field emergence, grain yield and fumonisin accumulation. The results varied with different treatments; the pure culture of *P. fluorescens* was more effective in reducing the incidence of *F. verticillioides* and increased the seed germination and vigour compared with the recommended carbendazim treatment in maize seeds. Similar results have been reported in sorghum[14] and in rice.[15] Several researchers have reported that application of fluorescent *Pseudomonas* to seed,[16] soil[17] and foliage[18–20] has also controlled several plant diseases.

Pure culture and formulations of *P. fluorescens* were also used under field conditions as seed treatment and spray treatment alone and in combination to evaluate seed quality parameters such as field emergence, ear rot disease, grain yield and level of fumonisin production for three consecutive years. The present results revealed that *P. fluorescens* significantly controlled ear rot disease and also improved field emergence and grain yield and reduced the level of fumonisins in maize grains.

Seeds treated with biological agents under field conditions were analysed for fumonisin production. The amount of fumonisins was significantly reduced throughout the treatments, but especially in seeds treated with pure culture of *P. fluorescens* as seed + spray treatment, compared with another seed treatment and the untreated control which showed high accumulation of fumonisin in seed samples. These experiments indicated that *P. fluorescens*

Table 6. Effect of *Pseudomonas fluorescens* treatments on fumonisin level in cultivars of maize[a,b]

Treatment	Total fumonisins (μg g^{-1}) Kanchan	Pioneer	Sweet corn
P. fluorescens **(10^9 cfu mL^{-1})**			
Seed treatment	81.4b	12.1b	128.07b
Foliar spray	92.2c	18.1c	167.40c
ST + FS	65.7a	08.3a	102.24a
Talc formulation 10 g kg^{-1}			
Seed treatment	96.5b	19.10b	189.32b
Foliar spray	99.2c	23.42c	207.10c
ST + FS	79.9a	12.55a	153.21a
Corn starch formulation 10 g kg^{-1}			
Seed treatment	116.4b	19.14b	189.26b
Foliar spray	123.4c	24.54c	212.30c
ST + FS	104.9a	14.77a	169.15a
Wheat bran formulation 10 g kg^{-1}			
Seed treatment	123.1c	23.23b	209.12b
Foliar spray	135.7d	26.92c	237.21c
ST + FS	119.4b	20.15a	201.18b
Carbendizim 2 g kg^{-1}	109.2a	22.43ab	186.65a
Control	234.0d	34.80d	878.45d

[a] Values are means from three repeat experiments with four replications and 100 seeds per replication in each experiment.
[b] Means designated with the same letter are not significantly different according to Tukey's HSD test at $P = 0.05$.

prevented fumonisin production by reducing the accumulation of *F. verticillioides* in maize.

A combined application of different formulations to seed and foliage was the most effective method for the control of disease in the field. Possibly both rhizosphere and phyllosphere populations of *P. fluorescens* helped to control disease. Both direct inhibition of the pathogen and systemically induced resistance in maize plants could be involved in control. Similar observations were also made in rice plants[21] in the case of *P. fluorescens*.

Increase in maize yield owing to *P. fluorescens* has been reported in several crops.[22–25] *Pseudomonas fluorescens* is known to produce several plant growth regulators such as gibberlins, cytokinins and indole acetic acid,[26,27] although effective control and yield increase appear to be dependent on method of treatment and the amount of biocontrol inoculum used. Lower disease incidence and resultant yield increase in seeds treated with microbial agents might be attributed to rapid multiplication of antagonists in the soil and its colonisation in the roots of seedlings, thereby preventing the establishment of the pathogens in the rhizosphere. The overall performance of *P. fluorescens* under field conditions was consistent during the study. In all 3 years of the study, seed treatment followed by foliar applications showed a considerable reduction in disease epidemics and fumonisin production compared with untreated seeds.

The present study demonstrated that *P. fluorescens* meets several criteria essential for an effective biocontrol agent. One feature is that the biocontrol agent must colonise the substrate or plant part targeted by the pathogenic organism. The inoculation of *F. verticillioides* and the isolated biocontrol agents into maize plants fulfilled another criterion, in that the biocontrol agent must be active under natural environmental conditions such as pH and temperature, so that growth of the biocontrol agent and antagonist coincide. However, previous studies have indicated that species of *Pseudomonas* may be more effective in certain ecological niches, such as specific soil types and temperature.[28,29] Further, the biocontrol potential for this isolate is more suited for toxin reduction in maize kernels intended for animal feed. The successful activity of the fungus on kernels in field and storage will depend on air, moisture and temperature requirements for this isolate. The present results provide the first evidence for activity of a *P. fluorescens* species as a suppressor of fumonisin synthesis. These results also support earlier reports that certain strains of *P. fluorescens* inhibit *F. verticillioides* growth.[30] The present study has shown that *P. fluorescens* is ecofriendly and much more effective against *F. verticillioides* and fumonisins and can be used as an alternative to fungicides to control toxigenic moulds.

REFERENCES

1 Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R, *et al*, Fumonisins, novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl Envl Microb* **54**:1806–1811 (1988).
2 Nelson PE, Desjardins AE and Plattner RD, Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. *Ann Rev Phytopathol* **31**:233–252 (1993).
3 Miller JD, Factors affecting the occurrence of fumonisin in corn. Abstracts of Papers of International Conference on the Toxicology of Fumonisin, Arlington, VA, pp. 21–22 (1999).
4 Prelusky DB, Trenholm HL and Savars ME, Pharmacokinetic fate of ^{14}C-labelled fumonisin B1 in swine. *Natural Toxins* **2**:73–80 (1994).
5 Thiel PG, Marasas WFO, Sydenham EW, Shephard GS and Gelderblom WCA, The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **117**:3–9 (1992).
6 Proceedings of the International Seed Testing Association, International Rules for Seed Testing. *Seed Sci Technol* **21**:25–30 (2003).
7 Patino B, Mirete S, Gonzalez-jaen MT, Mule G, Rodriguez MT and Vazquez C, PCR detection assay of fumonisin-producing *Fusarium verticillioides* strains. *J Food Prot* **67**:1278–1283 (2004).
8 Denning NJA, Morgan W, Whipps JM, Saunders JR and Winstanley C, The flagellin gene as a stable marker for detection of *Pseudomonas fluorescens* SBW25. *Lett Appl Microbiol* **24**:198–202 (1997).
9 Mortensen CN, *Seed Bacteriology Laboratory Guide*. Danish Government Institute of Seed Pathology for Developing Countries, Copenhagen, Denmark, pp. 1–58 (1992).
10 Baki AA and Anderson JP, Vigor determination in soybean seeds by multiple criteria. *Crop Sci* **13**:630–633 (1973).
11 Umesha S, Dharmesh SM, Shetty SA, Krishnappa M and Shetty HS, Biocontrol of downy mildew disease of pearl millet using *Pseudomonas fluorescens*. *Crop Prot* **17**:387–392 (1998).
12 Reid LM, Hamilton RI and Mather DE, *Screening Maize for Resistance to Gibberella Ear Rot*, Agriculture and Agri-Food Canada, Ottawa, Ont., Technical Bulletin 1996-5E, 2nd edition. John Wiley & Sons, Inc., New York, NY, pp. 1–28 (1996).
13 Gomez KA and Gomez AA, *Statistical Procedures for Agricultural Research*. John Wiley & Sons, Inc., New York, NY, pp. 1–42 (1984).
14 Raju NS, Niranjana SR, Janardhana GR, Prakash HS, Shetty HS and Mathur SB, Improvement of seed quality and field emergence of *Fusarium moniliforme* infected sorghum seeds using biological agents. *J Sci Food Agric* **79**:206–212 (1999).
15 Praveen Kumar L, Niranjana SR, Prakash HS and Shetty HS, Effect of *Pseudomonas fluorescens* formulation against *Pyricularia grisea* in Rice. *Crop Improvement* **27**:159–166 (2000).
16 Callan NW, Mathre DE and Miller JB, Biopriming seed treatment for biological control of *Pythium ultimum* pre-emergence damping-off in Sh2 sweet corn. *Plant Dis* **74**:368–372 (1990).
17 Hebbar P, Berge O, Heulin T and Singh SP, Bacterial antagonists of sunflower (*Helianthus annuus* L.) fungal pathogens. *Plant and Soil* **133**:131–140 (1991).

18 Clarkeson JP and Lucas J, Screening for potential antagonists of *Pseudocercosphorella harpotrichoides*, the causal agent of eye spot disease of cereals. *Plant Pathol* **42**:543–551 (1993).
19 Gnanamanickam SS and Mew TW, Biological control of blast disease of rice (*Oryza sativa* L.) with antagonistic bacteria and its mediation by a *Pseudomonas* antibiotic. *Ann Phytopathol Soc* **58**:380–385 (1992).
20 Praveen Kumar L, Niranjana SR, Prakash HS and Shetty HS, Improvement of seed quality and field emergence of rice seeds using an antagonistic strain of *Pseudomonas fluorescens*. *Asian J Microbiol Biotechnol Environ Sci* **3**:11–15 (2001).
21 Albouvette C, Lemanceau P and Steinberg C, Recent advances in the biological control of Fusarium wilts. *Pestic Sci* **37**:365–373 (1993).
22 Gamilel A and Katan J, Involvement of fluorescent *Pseudomonads* and other microorganisms in increased growth response of plants in solarised soils. *Phytopathology* **81**:474–502 (1991).
23 Vidyasekaran P and Muthamilan M, Development of formulation of *Pseudomonas fluorescens* for control of chickpea wilt. *Plant Dis* **79**:782–786 (1995).
24 Srinivas C, Niranjana SR and Shetty HS, Effect of bioagents and fungicides against *Phomopsis vexans* and on seed quality of Brinjal. *J Crop Improve* **32**:95–101 (2005).
25 Niranjan Raj S, Shetty NP and Shetty HS, Seed bio priming with *Pseudomonas fluorescens* isolates enhances growth of pearl millet plants and induces resistance against downy mildew. *Internat J Pest Manag* **50**:41–48 (2004).
26 Dubeikovsky AN, Mordukhova EA, Kochethov VV, Polikarpova FY and Boronin AM, Growth promotion of black currant soft woodcuttings by recombinant strain *Pseudomonas fluorescens* BSP53a synthesizing an increased amount of indole-3-acetic acid. *Soil Biol Biochem* **25**:1277–1281 (1993).
27 Lifshitz R, Kleopper JW, Kozlowski M, Simonson C, Cavison J, Tipping EM, *et al*, Growth promotion of Canola (rape seed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. *Can J Microbiol* **33**:390–395 (1987).
28 De Weger LA, van der Vlugt CIM, Wijfjes AHM, Bakker PAHM, Schippers B and Lugtenberg B, Flagella of a plant-growth stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J Bacteriol* **169**:2769–2773 (1987).
29 De Weger LA, Bakker PAHM, Schippers B, van Loosdrecht MCM and Lugtenberg B, *Pseudomonas* spp. with mutational changes in the O-antigenic side chain of their lipopolysaccharides are affected in their ability to colonize potato roots, in *Signal Molecules in Plant–Microbe Interactions*, ed. by Lugetnberg BJJ. Springer-Verlag, Berlin, Germany, pp. 197–202 (1989).
30 Calistru C, Mclean M and Berjak P, *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species: a study of the production of extracellular metabolites by *Trichoderma* species. *Mycopathologia* **137**:115–124 (1997).
31 Williams RJ and Singh SD, Control of pearl millet downy mildew by seed treatment with metalaxyl. *Ann Appl Biol* **97**:263–268 (1981).

