Research

Fungi Associated with Garlic During the Cropping Season, with Focus on *Fusarium proliferatum* and *F. oxysporum*

Letizia Mondani, Giorgio Chiusa, and Paola Battilani†

Università Cattolica del Sacro Cuore, Department of Sustainable Crop Production (DIPROVES), 29122 Piacenza, Italy

Accepted for publication 25 November 2020.

Abstract

Fusarium proliferatum has been reported as the main causal agent of garlic dry rot during the postharvest stage, but information on this fungus during the crop growth stage is lacking. We focused on the cropping season of garlic (*Allium sativum* L.) in the field, until its harvest, with the aim of clarifying the role of *F. proliferatum* in bulb infection as well as the impact of crop growing conditions on pathogen-plant interaction. Studies were conducted in Piacenza (northern Italy) for three seasons from 2016 to 2019. Six garlic farms were sampled. A different field was sampled every year. Soil samples were recovered at sowing time for the counting of fungal colony forming units (CFU). Plant samples were collected at three growth stages, from BBCH 15 (fifth leaf visible) to BBCH 49 (ripening), for which disease severity assessment and fungi isolations were performed. *Fusarium* was the most frequently isolated genus, of which *F. proliferatum* and *F. oxysporum* were the dominant species. *F. proliferatum* registered the highest incidence in all the farms tested, but *F. oxysporum* was dominant in the first year of the study. *F. oxysporum* incidence was correlated with dry weather, whereas *F. proliferatum* was correlated with rainy weather. In conclusion, our result confirms the association of *F. proliferatum* with garlic bulbs from the crop's early growth stages, suggesting potential seed transmission as a source of this fungal pathogen. Further studies should investigate the link between fusaria occurrence in the field and dry rot outbreaks occurring postharvest and during storage of garlic.

Keywords: *Allium sativum*, good agricultural practices, garlic dry rot, *Fusarium oxysporum*, *Fusarium proliferatum*, mycotoxins, *Penicillium*, water activity

Garlic (*Allium sativum* L.) is a globally grown crop, with 25 million tons of bulbs produced yearly. Although Asia contributes about 92% of global production, Europe contributes 3.4% (http://www.fao.org/faostat; data 2018), which includes high-quality niches of garlic production, sometimes called protected designation of origin (PDO) (Spagnoli 2014). Therefore, in some geographic areas, garlic is a very important crop for sustaining farmers' incomes.

In recent years, however, huge yield losses during postharvest, of up to 30% of production, have occurred because of garlic dry rot (Leyronas et al. 2018; Quesada-Ocampo et al. 2014; Tonti et al. 2012), whose main causal agent worldwide is reportedly *Fusarium proliferatum*.

During the storage phase, necrotic spots, centrally depressed, become visible as garlic dry rot's initial disease symptoms; in cases of severe fungal attacks and conducive conditions, the bulbs are hollowed out and white mycelium is sometimes seen (Dugan et al. 2003; Leyronas et al. 2018; Moharam et al. 2013; Palmero et al. 2010; Salvalaggio and Ridao 2013; Sankar and Babu 2012; Seefelder et al. 2002; Stankovic et al. 2007; Tonti et al. 2012).

†Corresponding author: P. Battilani; paola.battilani@unicatt.it

Funding: This work was partially funded by PSR program 16.1.01 "Gruppi operativi del PEI per la produttività e le sostenibilità dell'agricoltura" Sottomisura 16.1 of Emilia Romagna region, Focus Area 2A. Project N. 5005108, "Guidelines to reduce Fusarium rot in Piacenza white garlic".

The author(s) declare no conflict of interest.

F. proliferatum is a well-known plant pathogen, one able to cause diseases in many key agricultural crops, such as maize, wheat, barley, rice, asparagus, and pea during their cropping season (Alberti et al. 2018; Bargen et al. 2009; Dugan et al. 2003; Jurado et al. 2010; Kenényi Mulé et al. 2002; Palmero et al. 2012; Proctor et al. 2010; Stępień et al. 2011), but it can also be a pathogen of weeds (Bhale et al. 2012). The fungus *F. proliferatum* is a mycotoxin producer, mainly of fumonisins B_1 and B_2, compounds that are toxic to animals and humans (Cendoya et al. 2014; Desjardins 2006; Gálvez et al. 2017; Rheeder et al. 2002), for which strains' toxicity varies greatly depending on the plant species they are isolated from (Górna et al. 2016; Stępień et al. 2015). Other noted *Fusarium* species involved in garlic decay are *F. oxysporum* and *F. culmorum*, which mainly cause garlic basal plate rot and basal rot in field settings (Crowe et al. 1987; Matuo et al. 1986; Rengwalska and Simon 1986).

We could only find one report in the literature on the epidemiology of fusaria during garlic's growth stage in the field and its relationship to dry rot postharvest. Stankovic et al. (2007) found *F. proliferatum* to be the predominant species isolated from diseased garlic plants in Serbia during the cropping season there, yet they did not report the crop phenology of sampling, nor discuss its link to postharvest dry rot. Hence, information on fungi involved in postharvest garlic dry rot during crop development is surprisingly scarce, and details on the infection cycle of the causal agent are lacking. To fill this knowledge gap, this study focused on garlic's cropping season, until harvest, with three objectives: (i) to confirm the occurrence of *F. proliferatum* and other fungal species possibly involved in dry rot disease; (ii) to investigate the dynamic of

occurrence of these fungi in bulbs; and (iii) to evaluate the impact of soil inoculum, weather conditions, and agricultural practices on bulb infection.

Farm Location, Cropping System, and Meteorological Data

In three cropping seasons (2016 to 2019), six farms growing white garlic, located in the province of Piacenza (northern Italy), were selected based on their history of dry rot incidence during the prior 3 years reported by farmers (low = around 5%, and high = 15 to 20%, losses from dry rot). From the six farms, a different field in each was selected each year on account of crop rotation. All but one farm followed a 4-year crop rotation system avoiding Liliaceae; they included *Beta vulgaris*, *Phaseolus vulgaris*, *Glycine max*, *Medicago sativa*, *Solanum lycopersicum*, *Triticum vulgare*, and *Zea mays.* The crop cultivated before garlic was *T. vulgare* in all farms, except farm 6 in years 2 and 3, for which *P. vulgaris* and *Z. mays* were the previous crops, respectively. Only farm 4 followed the rotation of *A. sativum–T. vulgare–A. cepa–T. vulgare–Z. mays*.

Information regarding the soil texture and cropping system—seed treatment, sowing date, irrigation, fertilizer, and fungicide distribution—were collected for each farm. Hourly air temperature (°C), relative humidity (%), and rain (mm) were obtained from the agrometeorological service, Emilia Romagna region, based on a virtual grid of squares, each 5 km^2 wide; meteorological data are estimated on the base of all data available, intended as meteorological stations and radar located in each square (Bottarelli and Zinoni 2002). Some weather-derived variables were calculated, based on data collected: the sum of daily mean temperatures (Σ T mean), the sum of daily rainfall (Σ rain), the mean temperature of a given period (T mean), the mean of relative humidity of a given period (RH mean), the sum of degree days (SumDD), and the sum of rainfall (SumR). Each derived variable was computed for four time periods from garlic sowing to harvest according to growth stages reported by BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie): I = from sowing to 31 December; II = from 1 January to BBCH 15 (fifth leaf visible; around mid-April); III = from BBCH 15 to BBCH 45 (half of the bulb's diameter; last 10 days of May); and IV = from BBCH 45 to BBCH 49 (ripening; last days of June).

Figure 1 summarizes the study design used.

Soil Sampling and Quantification of Fungi

In late October of each year (2016 to 2018), soon after garlic sowing time, soil samples were collected at five sampling points along the diagonals of each selected field. Using a single Edelman auger combination type having a 5-cm diameter (Eijkelkamp, Giesbeek, Netherlands), three samples of soil were taken by digging through the ground section from the surface down to 50 cm, for a total volume of ~3,000 cm^3. All the farms were sampled on the same day. Soil samples were used to determine fungi colony-forming units per gram of soil (CFU/g).

To determine colony-forming units, 5 g of soil per sample was diluted in 45 ml of peptone water 0.1% and shaken for 30 min. Spread plate serial dilutions (10^{-2} to 10^{-5}) were made, in triplicate, adding 100 µl of suspension on *Fusarium* selective agar (composed of 1 liter of bidistilled water; 20 g of agar [Oxoid, Basingstoke, U.K.]; 20 g of dextrose; 0.5 g of KH_2PO_4; 2 g of $NaNO_3$; 0.5 g of $MgSO_4 \cdot 7\ H_2O$; 1 g of yeast extract; 1 ml/liter of 1% $FeSO_4 \cdot 7\ H_2O$; amended with 5 ml/liter of 2,6-dichloro-4-nitroaniline 1%; 0.1 g/liter of streptomycin sulfate; and 0.01 g/liter aureomycin sulfate). Plates were incubated at 25°C for 7 days with 12-h light photoperiod before being counted with a colony counter (CL-570, Sibata, Saitama, Japan). Dilutions of 10^{-2} to 10^{-4} were used for the counting.

Both the total CFU/g, intended to represent all fungal colonies grown, irrespective of the genus, and *Fusarium* spp. CFU/g were considered. Further, well-separated *Fusarium* spp. colonies, commonly observed in the dilution series of 10^{-3} to 10^{-4}, were transferred and identified at species level based on their morphology (Leslie and Summerell 2006).

Garlic Sampling and Characterization

Three garlic growth stages were monitored: BBCH 15 (fifth leaf visible; around mid-April), BBCH 45 (half of the bulb's diameter; last days of May), and BBCH 49 (ripening; last days of June) (Lopez-Bellido et al. 2016). In each growth stage, 20 plants were collected from each field at the five sampling points of its two diagonals, so 100 plants were collected yearly in each farm per growth stage (5,400 plants in total: 100 plants × 6 farms × 3 growth stages × 3 years). The bulb of each plant was used both for disease severity assessment and fungal isolations. When sampling the BBCH 49 growth stage, water activity (a_w) was also measured.

Disease severity. All the garlic plants were visually observed by the same individual and scored into one of five severity classes (Table 1). Then, a severity index was calculated by multiplying the number of plants attributed to each class of disease severity with the corresponding value of disease severity, for each sampling point (Table 1, Fig. 2). The mean disease severity/plant was computed for

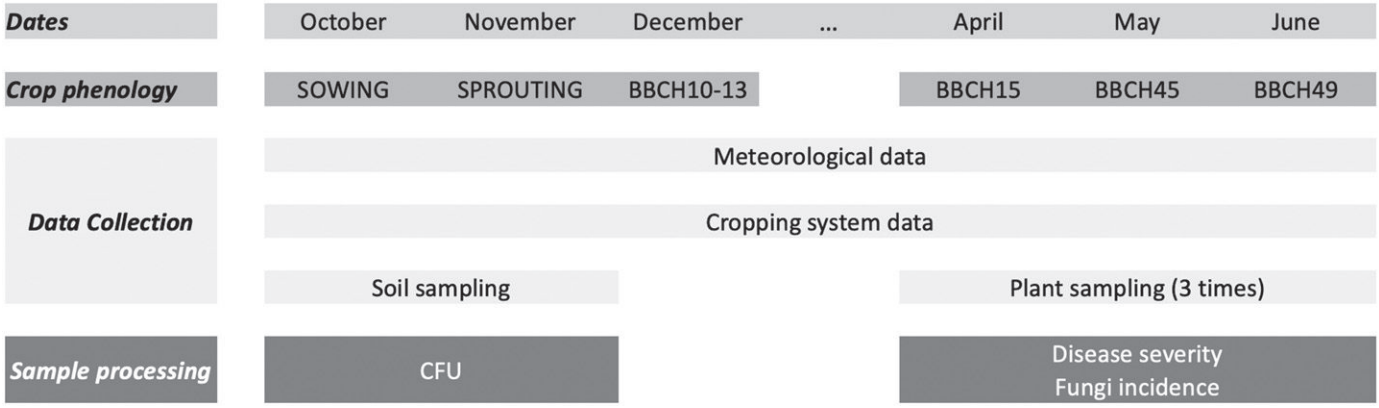

FIGURE 1

Seasonal garlic crop stages, data collection, and study design.

each sampling point as severity index/20 (20 = number of plants collected per sampling point).

Fungal isolations. A total of 25 plants per farm were randomly selected among each severity class observed; a portion of the basal plate including part of the symptomatic area (i.e., the area between the cloves and roots) was sampled from each plant. Tissue samples were washed under running tap water for 30 min, disinfected for 3 min in NaOCl 1%, rinsed thrice in sterile water, dried under a hood under sterile conditions, plated in water agar (1 liter of bidistilled water, 20 g of agar, and 250 ppm streptomycin sulfate). The water agar plates were then incubated at 25°C, with a 12-h light photoperiod. After 5 days of incubation, growing colonies were transferred to potato dextrose agar (1 liter of potato broth, 200 g of potato/liter of water; 15 g of agar; and 10 g of dextrose). The colonies were grouped according to their morphological characters and identified at the genus level (Schwartz and Mohan 2016). Next, those colonies attributed to *Fusarium* spp. were identified at the species level based on their morphological characters (Leslie and Summerell 2006). Four isolates per crop stage per year belonging to either *F. proliferatum* or *F. oxysporum* were randomly selected (total of 36 isolates) for further confirmations. The 36 isolates were further purified by single-spore isolations and then used for molecular confirmation using methods described by Mbofung and Pryor (2010) and Nicolaisen et al. (2009). DNA extractions were carried out using the commercial extraction kit Nucleo Spin Plant II (Macherey-Nagel, Düren, Germany). A nested polymerase chain reaction (PCR) was performed to identify *F. oxysporum*, using the primers GYCF1 (5′-CTCCGGATTTCTGGAGACTTG-3′)/GYCR4C (5′-ACTATCGTGTGCCGGGGTTGGC-3′) and GYCF1 (5′-CTCC GGATTTCTGGAGACTTG-3′)/R943 (5′-CCCATACTATATAC CAGACG-3′), in a 50-µl volume (25 µl of GoTaq G2 PCR 2X MM (Promega, M7833), 0.3 µl of each primer, 3 µl of DNA, and ultrapure water to volume). The PCR conditions were as follows: 3 min at 94°C, 30 cycles of 30 s at 94°C, 2.24 min at 65°C, 1 min at 72°C, with an elongation step of 10 min at 72°C. These results were read on a 1.5% agarose gel with a Gene Ruler 1-kb DNA Ladder (SM0313, Thermo Fisher Scientific).

The *F. proliferatum* isolates were identified through real-time PCR in a total reaction volume of 20 µl (10 µl of GoTaq G2 PCR 2X MM [Promega, A6101], 0.4 µl each of the primers F.pro 220F [5′-CTTCGATCGCGCGTCCT-3′] and F.pro 270R [5′-ACGTTTC GAATCGCAAGTG-3′], 0.4 µl of $MgCl_2$ [Promega, A6101], 0.33 µl of CXR [Promega, A6101], and ultrapure water to volume). The PCR reactions followed these steps: 10 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C, with the melting curve analysis performed from 60 to 95°C, using a 0.3°C/30 s increment.

Water activity. Water activity (a_w) was measured using Aqualab Pre instruments (Meter Group, Pullman, WA). Central asymptomatic cloves belonging to five plants from two sampling points within the same farm were used for this analysis. The measurements were taken at harvesting in all the farms and years, in triplicate.

Data analysis. CFU/g were ln transformed; disease severity in bulbs and incidence of fungi/*Fusarium* spp. in bulbs were arcsine transformed to homogenize means (Clewer and Scarisbrick 2001). After transformation, analysis of variance was performed separately for fungal CFU/g in soil, disease severity in bulbs, incidence of fungi/*Fusarium* spp. in bulbs, and bulbs' a_w. Tukey's test was used to compare means and highlight significant differences between them. Pearson correlations were applied to meteorological data (SumDD and SumR), disease severity at BBCH 49, and incidence of *Fusarium* spp. from BBCH 15 to BBCH 49. All statistical analyses were performed with the package PASW statistics (version 19, SPSS, Chicago, IL).

Cropping System Applied by Farmers

The eastern section of the Po valley in the province of Piacenza, Italy, where the six farms included in the study were located, is characterized by heavy soils. According to the USDA soil triangle, farms 1 and 2 had a clay composition, farms 3 and 6 were classified as clay loam, and farms 4 and 5 were classified as silty clay loam, based on the soil analysis managed by the farmers (Owens and Rutledge 2005).

Fertilizers were applied before sowing and at the BBCH 15 growth stage of garlic. Only farms 1 and 5 applied both organic and inorganic fertilizers. Chemical fertilizers were applied at different doses each year, based on soil composition and crop rotation

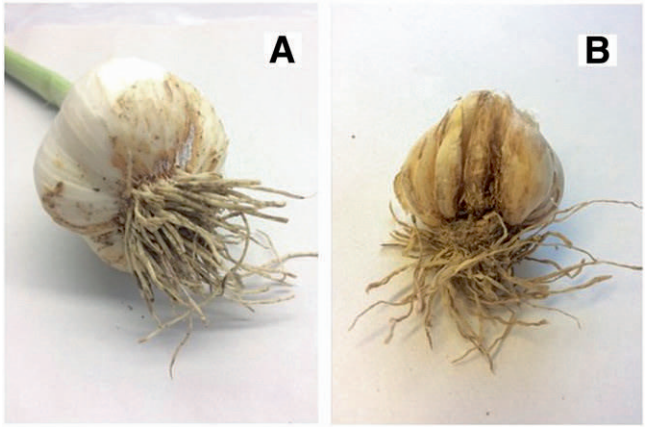

FIGURE 2
Examples of garlic bulbs attributed to disease severity class 3 (**A**) and 4 (**B**) at growth stage BBCH 49.

TABLE 1
Assessment scale applied to quantify garlic bulb disease severity in the field study

Severity symptoms	Class	Disease severity (%)
No symptoms	0	0
Small brown spots near the basal plate (base of radicles)	1	10
Brown spots on half of the basal plate	2	35
Brown spots on the whole perimeter of the basal plate, sometimes growing mycelium visible	3	65
Brown spots on the basal plate extended to the bulb and white mycelium visible	4	90

according to the Regional Rules for Integrated Production as stated in the document "Disciplinare di produzione integrata della regione Emilia Romagna" (Regione Emilia-Romagna 2019), albeit with some restrictions implemented according to European Union legislation.

All the producers sowed garlic, Ottolini variety, in late October, using a seed density of 270,000 cloves/ha. The mentioned Regional Rules include a *Trichoderma* seed treatment, and three producers (farms 2, 5, and 6) applied this biological seed coating in 2018. During the cropping season fungicides were applied from BBCH 45 onward until harvest; the active ingredients listed in the Regional Rules belong to the triazole, carbamate, and strobilurin families. Only farm 3 applied azoxystrobin, whereas all the other producers used at least one treatment of tebuconazole, reportedly effective for fusaria control, and also applied other active ingredients (dimethomorph + pyraclostrobin).

Irrigation varied between years, depending on rainfall events in May and June; the number of irrigations ranged from zero to four but were similar between farms in the same year.

Occurrence of Fungi in Soil

Generally, the dominant genus isolated from the soil was *Fusarium*, followed, in descending order, by *Rhizopus*, *Trichoderma*, and *Penicillium.* Significant differences were noticed between years, in terms of the overall colony counts for the total number of CFU/g of soil and *Fusarium* CFU/g of soil, and both were higher in year 1 than the other two years; however, the order of magnitude remained the same (10^3). No significant differences were detected among farms in either their total CFU/g or *Fusarium* CFU/g (Table 2). Two dominant species were identified in *Fusarium* genera, *F. oxysporum* and *F. proliferatum*, both with colonies on the order of 10^3 CFU/g; the other *Fusarium* species identified (*F. solani*, *F. culmorum*, *F. semitectum*, and *F. equiseti*) globally contributed on the order of 10^2 CFU/g (data not shown).

Disease Severity and Occurrence of Fungi in Garlic Bulbs

Disease symptoms were only visible on the basal plate of bulbs. According to the assessment scale described in Table 1, disease severity varied significantly between farms, despite the limited range of variation (Table 3). Farms 1 and 6 incurred the least disease severity, whereas it was greatest in farms 3 and 4 (mean severity/plants: 41 versus 44%), and the differences were statistically significant. The impact of the garlic growth stage at sampling was also significant: disease severity increased about 10-fold from BBCH 15 to BBCH 45.

Regarding occurrence of fungi, the main genus isolated during the analysis of garlic bulbs was *Fusarium*, followed by *Penicillium*, although the latter occurred at a very low incidence (*Penicillium* spp. incidence <4%). No significant difference in incidence of fungi was detected between plants belonging to different symptomatic classes; therefore, this factor was excluded in the data analysis and discussion of results. *Fusarium* spp. were isolated with the highest incidence in farms 1, 2, and 4, whereas farm 3 scored the lowest incidence. Among the cropping seasons, years 1 and 3 showed a similar *Fusarium* spp. incidence (80.3 and 80.9%, respectively), which exceeded that of year 2 (71.5%) During the cropping season, there was a higher incidence of *Fusarium* at garlic growth stage BBCH 15 (77.6%) and BBCH 45 (82.5%) than at its harvest (71.4%); *Penicillium* spp. were isolated more frequently in year 1 (3.9%) and in BBCH 15 (3.9%) when compared with other years and growth stages (Table 3).

Concerning the *Fusarium* spp. isolates, two main species were identified, *F. oxysporum* and *F. proliferatum*, both confirmed by the molecular approach. Because *F. culmorum* (mean < 1.5%), *F. solani*, and *F. acuminatum* (mean < 0.2%) were sporadically isolated, they were not included in the data analysis. *F. oxysporum* was isolated less frequently in farm 3 (16.3%), with all five other farms characterized by a similar incidence of it (31.2 to 34.6%), whereas for *F. proliferatum*, the lowest incidence was in farm 6 (39.3%) and the highest in farm 4 (62.1%; Table 3).

With respect to differences between years, the incidence of *F. oxysporum* was halved going from year 1 to years 2 and 3, whereas *F. proliferatum* almost doubled its incidence in years 2 and 3 relative to year 1. Further, both *Fusarium* species were isolated more frequently at BBCH 15 and declined moving toward the garlic crop harvest. *F. oxysporum* started decreasing in frequency from BBCH 45, whereas *F. proliferatum* showed similar incidence at BBCH 15 and BBCH 45 but decreased at BBCH 49.

The interaction between years and farms was also relevant; in farms 4 and 5, *F. proliferatum* incidence was around 50% in all the three years considered, whereas in the other farms *F. proliferatum* doubled (farms 1, 2, and 6) or even tripled (farm 3) its incidence going from year 1 to year 3. By contrast, *F. oxysporum* incidence showed comparable results in all the farms except for farm 3, which had the lowest incidence in any sampled year (Fig. 3). Moreover, the incidence of *F. proliferatum* varied widely in different cropping seasons depending on the farm (Fig. 4). In year 1, *F. proliferatum* was isolated most frequently at BBCH 15 and diminished onward to the crop harvest, with the exception of farm 3, which showed the opposite pattern. In year 2, in farms 1, 3, and 4 the incidence of *F. proliferatum* diminished from BBCH 15 to BBCH 49, whereas in farms 2, 5, and 6 it was isolated more frequently at BBCH 45 (mean = 66%). Finally, in year 3, four farms out of the six registered their highest incidence of *F. proliferatum* at the BBCH 45 stage of garlic (farms 1, 3, 4, and 5; mean = 75%), whereas farms 6 and 2 showed opposing trends, in that one decreased (farm 6, from 68 to 48%) and one increased (farm 2, from 66 to 73%) in approaching the harvest time.

Regarding a_w, its mean value in garlic cloves at harvest was 0.978. Significant differences were detected between years, with a_w

TABLE 2
Mean CFU/g of all fungi (total) and *Fusarium* counted in soil samples collected over 3 years in six farms at the beginning of the garlic cropping season[z]

Factors	Total CFU/g of soil	*Fusarium* CFU/g of soil
Year	*	*
1	9.8×10^3 b	2.8×10^3 b
2	3.3×10^3 a	1.4×10^3 a
3	3.4×10^3 a	1.5×10^3 a
Farm	n.s.	n.s.
1	7.4×10^3	3.2×10^3
2	4.2×10^3	1.5×10^3
3	6.3×10^3	1.6×10^3
4	6.8×10^3	2.7×10^3
5	5.8×10^3	1.4×10^3
6	2.6×10^3	9.3×10^2

[z] Sampling year and farm were considered as factors in the ANOVA, and factor significance was reported before means (* indicates $P < 0.05$; n.s. = not significant). Different letters indicate significant differences according to Tukey's test ($P < 0.01$).

rising from year 1 to year 3 (a_w = 0.888, 0.915, and 0.937, respectively) and also differing between farms; farms 3 and 6 featured the lowest a_w, whereas farm 2 registered the highest value (0.896 and 0.929, respectively).

Meteorological Data and Their Effect on Fungal Incidence

Over the three years of this study, different combinations were recorded in the SumDD and SumR. Year 1 was characterized by high temperatures and few rainfall events (means: 2,115 SumDD, 188 mm of rain); year 2 was hot and rainy, with 2,207 SumDD and 369 mm of total rain; conversely, year 3 was the coldest (mean: 1,967 SumDD) and similar to year 2 in terms of rainfall (mean: 360 mm). In particular, year 3 was characterized by low temperatures and heavy rainfall in April and May (respective means: 14°C and 239 mm of rain). Full details on this seasonal variation in weather are given in Table 4.

Pearson correlations highlighted that *F. proliferatum* was most common at BBCH 15, around April, which was positively correlated to its incidence throughout the cropping season. Yet for *F. oxysporum*, the only positive correlation was between its incidence at BBCH 45 and BBCH 49 (r = 0.854**). The two dominant *Fusarium* species isolated are inversely related and displayed a

TABLE 3
Mean disease severity assessed on garlic basal plates and mean incidence of main fungal species isolated from basal plates[z]

Parameter	Disease severity (%)	Incidence (%)			
		Fusarium spp.	*F. oxysporum*	*F. proliferatum*	*Penicillium* spp.
Farm (F)	**	**	**	**	n.s.
1	40.4 a	82.3 c	33.9 b	54.4 bc	1.3
2	42.2 bc	84.5 c	32.4 b	56.7 bc	1.0
3	43.4 c	62.8 a	16.3 a	47.5 ab	1.8
4	43.7 c	84.8 c	31.2 b	62.1 c	2.9
5	42.7 bc	78.2 bc	34.7 b	50.1 ab	1.7
6	41.0 ab	70.7 ab	33.6 b	39.3 a	3.2
Year (Y)	n.s.	**	**	**	**
1	43.1	80.3 b	57.0 c	33.8 a	3.9 b
2	41.9	71.5 a	24.0 b	53.6 b	1.8 a
3	41.8	80.9 b	17.5 a	63.0 c	0.8 a
Growth stage (GS)	**	**	*	**	**
BBCH 15	4.9 a	77.6 b	36.0 b	54.4 b	3.9 b
BBCH 45	58.9 b	82.5 b	27.3 a	55.7 b	0.8 a
BBCH 49	62.9 c	71.4 a	28.4 a	44.9 a	1.4 a
Interaction					
F × Y	**	**	**	**	*
Y × GS	*	**	**	**	*
F × GS	**	**	**	**	**
F × Y × GS	**	**		**	*

[z] Garlic samples were collected in six farms over three cropping seasons at three growth stages (BBCH 15, BBCH 45, and BBCH 49). Farm, year, and growth stage were considered as factors in the ANOVA, and factor significance was reported before means. Different letters indicate significant differences according to Tukey's test ($P < 0.01$). ** indicates $P < 0.01$; * indicates $P < 0.05$; n.s. = not significant.

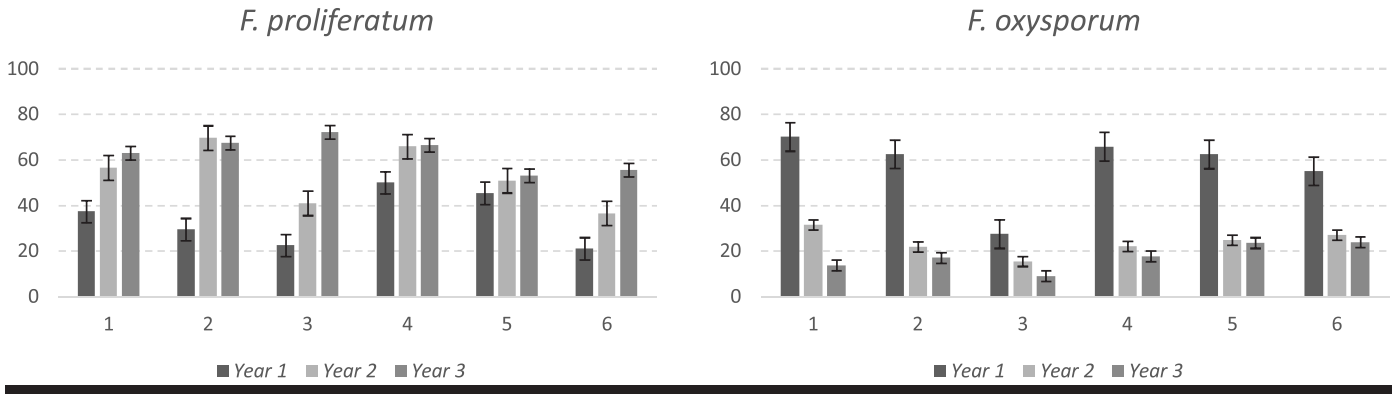

FIGURE 3
Incidence of the two main species of *Fusarium* isolated from garlic basal plates in the three years (2017 to 2019) they were sampled, from six farms. Bars are the mean ± SE.

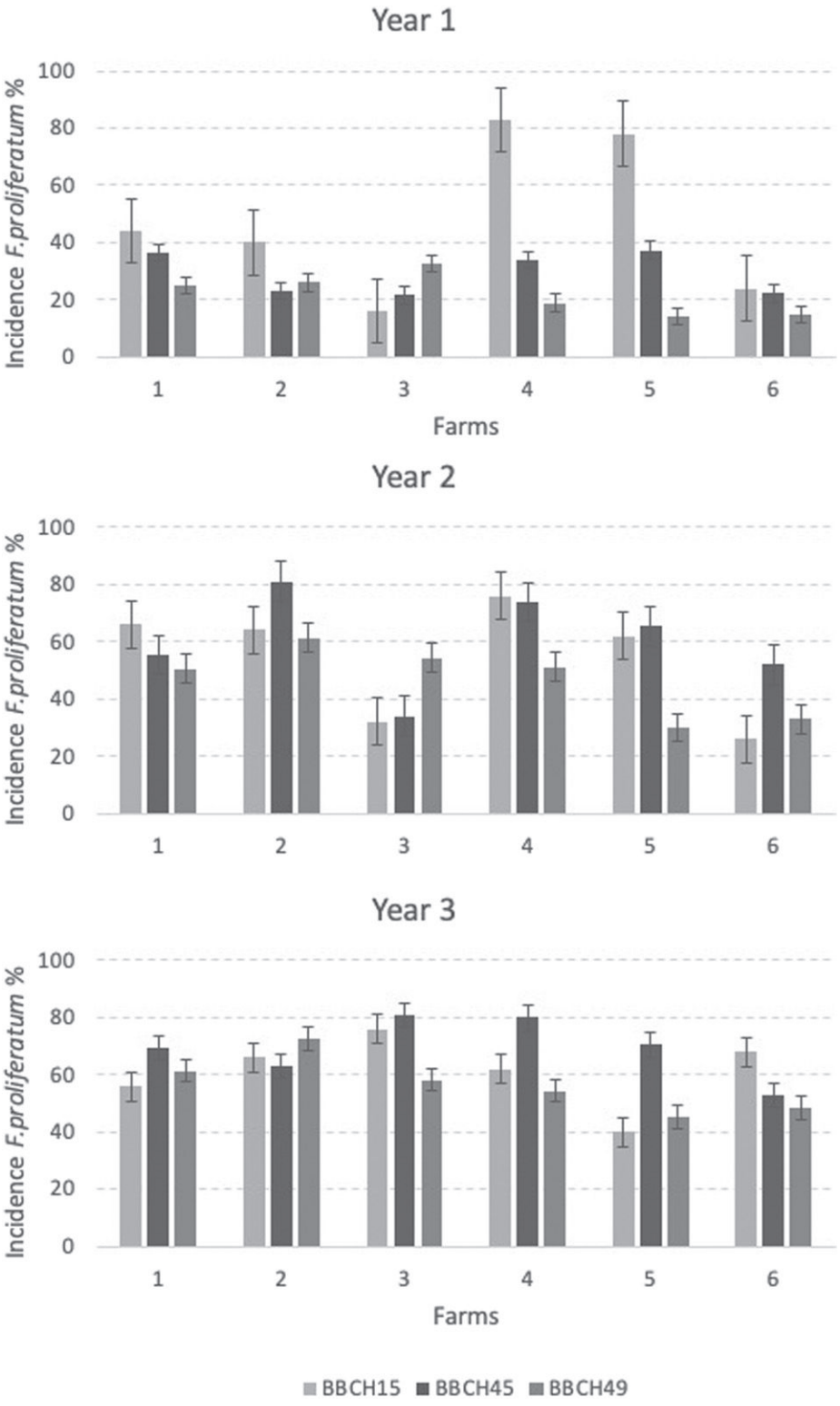

FIGURE 4

Incidence of *Fusarium proliferatum* isolated from garlic basal plates during the three years (2017 to 2019) they were sampled, from six farms, at three growth stages (BBCH 15, BBCH 45, and BBCH 49). Bars are the mean ± SE.

complementary incidence throughout the garlic cropping season, as suggested by their significant negative correlation at harvest (r = –0.694). Supporting this interpretation, rainy seasons were more favorable to *F. proliferatum* (r = 0.765), whereas *F. oxysporum* was isolated at its highest incidence in the driest season (i.e., year 1; Table 5). Correlations of disease severity at harvest with either *F. proliferatum* or *F. oxysporum* incidence were not significant.

Implications of Biotic and Abiotic Factors on *F. proliferatum* Occurrence in Garlic and Future Directions

Garlic dry rot, an emerging postharvest disease of garlic globally, has been a major concern for garlic growers in the province of Piacenza in northern Italy in recent years. At the postharvest stage, the available ways to control diseases are generally limited, being mostly based on managing temperature in storage sheds to ensure unfavorable conditions for fungal growth. Garlic is generally stored at –4°C. Therefore, preventing disease occurrence in the field is necessary by following the principle of integrated pest management and sustainable crop production (Sharma and Sharma 2005). This study was planned to fill some gaps in knowledge regarding the relevance of the crop growing period on the occurrence of *F. proliferatum* and other fungal species possibly involved in dry rot disease.

Two main *Fusarium* spp. were isolated from soil and garlic bulbs in this study, *F. proliferatum* and *F. oxysporum*, in agreement with literature reports (Dugan et al. 2003; Leyronas et al. 2018; Matuo et al. 1986; Palmero et al. 2010; Rengwalska and Simon 1986; Tonti et al. 2012). *Fusarium* inoculum occurred in soil; it was detected at comparable concentrations in all six farms and during the three successive years of sampling (about 10^3 CFU/g of soil), with *F. proliferatum* and *F. oxysporum* as the dominant species. The stability in *Fusarium* inoculum in soil suggests that the soil microbiota

TABLE 4
Meteorological data collected during three garlic cropping seasons in the areas of interest[y]

	2016–2017				2017–2018				2018–2019			
Meteo square – farm	I[z]	II	III	IV	I	II	III	IV	I	II	III	IV
450 – farm 2												
Σ T mean		753.1	644.2	809.7	599.3	795.9	736.9	723.3	571.0	640.3	815.7	584.0
Σ rain		76.7	62.7	22.3	185.3	216.6	86.4	49.5	221.1	117.7	247.7	6.1
T mean		7.3	15.8	24.5	7.4	7.0	18.9	23.3	7.8	6.5	14.8	24.3
RH mean		69.6	63.3	54.6	78.0	77.3	71.0	60.4	84.3	66.5	70.8	56.4
SumDD				*2,207.0*				*2,256.1*				*2,040.0*
SumR				*161.7*				*352.5*				*371.5*
491 – farm 4												
Σ T mean		723.7	634.4	802.0	354.1	771.9	746.4	716.5	560.2	615.3	800.4	578.1
Σ rain		76.2	61.9	27.2	190.6	241.4	82.8	50.0	233.4	126.8	258.3	6.9
T mean		7.0	15.5	24.3	5.4	6.8	18.7	23.1	7.7	6.2	14.6	24.1
RH mean		73.0	67.0	58.3	82.8	80.8	74.7	64.4	87.4	69.9	75.7	60.5
SumDD				*2,610.1*				*2,234.8*				*1,993.8*
SumR				*165.3*				*374.2*				*392.0*
530 – farms 5 and 6												
Σ T mean	567.8	659.2	621.6	782.2	518.7	724.4	714.0	709.9	609.8	534.7	777.2	568.5
Σ rain	86.8	76.2	114.3	32.3	147.0	226.4	89.8	52.4	217.3	112.1	227.3	2.3
T mean	6.8	6.4	15.2	23.7	6.5	6.4	18.3	22.9	7.9	5.4	14.1	23.7
RH mean	92.7	76.8	69.7	63.0	84.1	85.1	79.7	68.2	90.2	75.1	80.7	64.8
SumDD				*2,063.1*				*2,148.3*				*1,880.4*
SumR				*222.8*				*368.6*				*341.7*
531 – farm 1												
Σ T mean		659.2	621.6	782.2	400.9	765.8	702.7	713.4	479.4	570.2	789.1	573.9
Σ rain		76.2	95.6	32.3	147.0	240.6	81.6	50.9	225.3	118.3	242.9	5.7
T mean		6.4	15.2	23.7	5.6	6.7	18.5	23.0	7.0	5.8	14.3	23.9
RH mean		76.8	69.7	63.0	85.6	83.1	77.8	66.6	90.8	72.9	78.5	62.8
SumDD				*2,063.1*				*2,181.8*				*1,933.1*
SumR				*204.1*				*373.1*				*366.9*
568 – farm 3												
Σ T mean		672.1	624.7	786.2	594.2	748.8	738.8	723.7	675.3	577.9	816.8	590.4
Σ rain		59.9	95.1	28.5	160.3	214.2	97.1	66.4	226.8	106.4	216.5	3.3
T mean		6.5	15.2	23.8	7.1	6.6	18.9	23.3	8.4	5.8	14.9	24.6
RH mean		75.7	69.2	61.8	82.9	83.0	75.5	65.7	87.7	72.4	76.0	6.7
SumDD				*2,082.9*				*2,211.2*				*1,985.1*
SumR				*183.5*				*377.7*				*326.2*

[y] The agrometeorological service for Emilia Romagna region is based on a virtual grid of squares (meteo square), each 5 × 5 km. Meteorological data are estimated on the basis of all data available, intended as meteorological stations and radar located in each square (Bottarelli and Zinoni 2002). Time periods: I = from sowing to 31 December; II = from 1 January to BBCH 15; III = from BBCH 15 to BBCH 45; and IV = from BBCH 45 to BBCH 49. Σ T mean = the sum of daily mean temperatures; Σ rain = the sum of daily rainfall; T mean = the mean temperature of a given period; RH mean = the mean of relative humidity of a given period; SumDD = the sum of degree days; and SumR = the sum of rainfall. As an aid to readers, values for SumDD and SumR for the growing season are italicized.

[z] In the first 3 months of the project (October to December 2016), we only recovered data from one weather station with a maximum distance of 15 km from the sampled fields.

plays a minor role in bulb infection occurrence, at least in the area under study; in fact, the incidence of these fungi in bulbs depended on the farm and growing year, irrespective of the stability of *Fusarium* in soil. The low variability of fusaria in soil was expected, given the small area sampled overall (around 50 km^2) and the 4-year crop rotation avoiding Liliaceae that was followed by almost all the farms we surveyed. This latter choice reduces the capacity of fusaria to survive prolonged periods in soil, as chlamydospores or on crop residues (Cotten and Munkvold 1998; Schwartz and Mohan 2016), which would explain its similar prevalence between farms and years.

In this study, brown-tan spots, related to *Fusarium* spp. infection in garlic bulbs, were first observed at early growth stages (BBCH 15) almost exclusively on the basal plate. The severity of symptoms increased as the cropping season progressed, and at BBCH 49 almost every plant sampled had visible symptoms, yet only a few plants were recorded as belonging to severity class 4, therefore with symptoms on bulbs. Severity classes were not significant for incidence of fungi in the symptomatic samples plated, and it was possible to recover *Fusarium* spp. from basal plates irrespective of the severity class attributed.

The incidence of *F. proliferatum* at BBCH 15 correlated with its incidence detected in the two following growth stages, whereas a correlation in the incidence of *F. oxysporum* was only found between BBCH 45 and BBCH 49. This suggests early stages are highly relevant for *F. proliferatum* in garlic, perhaps related to cloves serving as the inoculum source (Dugan et al. 2007, 2019), whereas weather conditions during the cropping season could play a major role in the incidence of *F. oxysporum*.

In this 3-year study, *F. proliferatum* was the most common fungus in all the farms, having a mean of 50.1% versus 32.8% for *F. oxysporum*, but the latter had a higher incidence in year 1; this was significantly correlated to weather conditions, year 1 being drier than either of the two years that followed it. Further, the two *Fusarium* species were inversely related, showing a complementary incidence, and rainy weather seems to foster the proliferation of *F. proliferatum*. In previous research that compared the species' spore germination tested at different a_w, drought conditions were tolerated better by *F. oxysporum*, whose conidial germination was observed until a_w = 0.88, whereas *F. proliferatum* germination stopped at a_w = 0.90 (El Halouat and Debevere 1997; Marín et al. 1996). Water activity of garlic bulbs at harvest changed between years in this study, moving from a_w = 0.888 in year 1, which was warm and dry, to a_w = 0.937 in year 3, being mild and rainy, thus corroborating *F. oxysporum* as better adapted to drought conditions. Further, a_w measured at harvest is commonly still useful for these two *Fusarium* species that probably stay active, at least during the first period of natural drying postharvest.

In Serbia, the predominant species isolated from garlic and onions during their cropping season was *F. proliferatum*, whereas *F. oxysporum* was rarely found in association with basal plate rot (Stankovic et al. 2007). But a survey of garlic seeds in the United States and China demonstrated that both *Fusarium* species are present in propagation material and capable of causing symptoms on garlic seedlings (Dugan et al. 2007). No weather data were reported in either study above; therefore, it is not possible to confirm if the differences reported are due to the meteorological conditions or to any other biotic or abiotic factor not reported by the authors.

Penicillium spp. were also isolated from symptomatic plants, and this fungus has been recognized as a possible causal agent of blue mold and bulb decay in garlic (Dugan et al. 2014; Schwartz and Mohan 2016), yet never associated with garlic dry rot. Its low incidence was noted in our studied area (always <4%), so we tentatively conclude it is not a predominant pathogen of garlic at the field-cultivation stage in our climatic zone and likely not involved in garlic dry rot.

Finally, in considering the influence of the cropping system used, the minor differences between farms in this respect were never related to incidence of fusaria on basal plates. All farms were characterized by heavy soils, and the maximum amount of fertilizers that can be distributed on the crop is fixed by law, so the final effect on garlic was comparable between farms. Moreover, all control strategies were applied according to regional protocol for integrated production, so quantities and products were almost the same in all farms and meant to achieve the same result in plant protection, so this factor is probably irrelevant to garlic dry rot in this context.

To conclude, this is the first study of field-cultivated garlic, with soil analysis and plant sampling made in successive growth stages, from BBCH 15 to BBCH 49. During the cropping season, *Fusarium* was the predominant genus isolated from basal plate tissue in garlic plants, with *F. proliferatum* clearly the dominant species. Therefore, this confirms the association of *F. proliferatum* with garlic bulbs during the cropping season and its possible role in garlic dry rot postharvest. *Fusarium* species were isolated from the first stage of the garlic crop sampled, and the inoculum in the soil seems to

TABLE 5
Pearson correlation coefficients computed for data collected during the 3-year study of six farms growing garlic in Piacenza province[z]

Parameter	FP 2	FP 3	FP 4	FO 2	FO 3	FO 4	SumDD	SumR
Severity	0.020	0.100	–0.215	0.102	0.216	0.285	–0.061	–0.413
FP 2		**0.512***	0.245	0.082	–0.094	–0.055	–0.018	0.205
FP 3			**0.737****	–0.255	**–0.766****	**–0.666****	–0.220	**0.773****
FP 4				**–0.569***	**–0.837****	**–0.694****	–0.154	**0.765****
FO 2					0.467	0.336	**0.575***	–0.261
FO 3						**0.854****	0.041	**–0.876****
FO 4							0.085	**–0.886****
SumDD								–0.101

[z] Severity = disease severity at growth stage BBCH 49; FP 2 = incidence of *Fusarium proliferatum* at growth stage BBCH 15; FP 3 = incidence of *F. proliferatum* at growth stage BBCH 45; FP 4 = incidence of *F. proliferatum* at growth stage BBCH 49; FO 2 = incidence of *F. oxysporum* at growth stage BBCH 15; FO 3 = incidence of *F. oxysporum* at growth stage BBCH 45; FO 4 = incidence of *F. oxysporum* at growth stage BBCH 49; SumDD = the sum of degree days; and SumR = the sum of rainfall. As an aid to readers, significant correlations (** indicates $P < 0.01$, and * indicates $P < 0.05$) are bolded.

have little if any relevance. Accordingly, further investigations should consider instead the role of garlic seed, which commonly means the cloves obtained in the preceding year of production, as a source of inoculum, as suggested by Dugan et al. (2007).

Based on these results, because incidence of fungi was not related to symptom severity, bulbs infected by *F. proliferatum* can enter the postharvest stage. This supports the relevance of prevention measures to mitigate yield losses due to garlic dry rot in the postharvest stage. More care in garlic seed selection, as well as best practices for bulb sanitation to reduce/exclude seeds as possible inoculum sources, ought to be considered by garlic farmers. Lastly, further studies regarding dry rot outbreak postharvest are required to link field data on *F. proliferatum* occurrence and to confirm the role of the preharvest phase in postharvest disease outbreaks.

Acknowledgments

Letizia Mondani worked on this project as a PhD student, at the Agrisystem PhD school, funded by the PSR program 16.1.01 "Gruppi operativi del PEI per la produttività e le sostenibilità dell'agricoltura" Sottomisura 16.1 of Emilia Romagna region, Focus Area 2A. Project N. 5005108, "Guidelines to reduce *Fusarium* rot in Piacenza White Garlic". Mariachiara Gerace contributed to the laboratory analysis as part of her master's thesis. Cinzia Colombi assisted with the molecular laboratory analysis. The authors thank the agrometeorological service of the Emilia Romagna region for providing the meteorological data.

Literature Cited

Alberti, I., Prodi, A., Montanari, M., and Paglia, G. 2018. First report of *Fusarium proliferatum* associated with *Allium fistulosum* L. in Italy. J. Plant Dis. Prot. 125:217-219.

Bargen, S. v., Martinez, O., Schadock, I., Eisold, A. M., Gossmann, M., and Büttner, C. 2009. Genetic variability of phytopathogenic *Fusarium proliferatum* associated with crown rot in *Asparagus officinalis*. J. Plant Physiol. Pathol. 157:446-456.

Bhale, U. N., Chatage, V. S., and Ambuse, M. G. 2012. First report of *Fusarium proliferatum* inciting wilt of *Rumex acetosa* L. in Maharashtra, India. J. Plant Pathol. Microbiol. 03:2020.

Bottarelli, L., and Zinoni, F. 2002. La rete meteorologica regionale. Divulgatore 5:13-17.

Cendoya, E., Monge, M. P., Palacios, S. A., Chiacchiera, S. M., Torres, A. M., Farnochi, M. C., and Bourguignon, E. 2014. Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. Food Control 37: 56-61.

Clewer, A. G., and Scarisbrick, D. H. 2001. Page 332 in: Practical Statistics and Experimental Design for Plant and Crop Science. Wiley, Oxford, U.K.

Cotten, T. K., and Munkvold, G. P. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. Phytopathology 88: 550-555.

Crowe, F. J., Koepsell, P., Stahl, S., Brevig, R., Greathead, A. S., and Kurtz, E. A. 1987. Continued investigation into the epidemiology and control of Fusarium bulb rot of garlic. Pages 34-46 in: Central Oregon Agricultural Research Center 1987 Annual Report. Oregon State University, Corvallis, OR.

Desjardins, A. E. 2006. *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology. American Phytopathological Society, St. Paul, MN.

Dugan, F. M., Hellier, B. C., and Lupien, S. L. 2003. First report of *Fusarium proliferatum* causing rot of garlic bulbs in North America. Plant Pathol. 52: 426.

Dugan, F. M., Hellier, B. C., and Lupien, S. L. 2007. Pathogenic fungi in garlic seed cloves from the United States and China, and efficacy of fungicides against pathogens in garlic germplasm in Washington State. J. Phytopathol. 155:437-445.

Dugan, F. M., Lupien, S. L., and Hellier, B. C. 2019. Infection by *Fusarium proliferatum* in aerial garlic bulbils is strongly reduced compared to rates in seed cloves when both originate from infected bulbs. Crop Prot. 116: 43-48.

Dugan, F. M., Lupien, S. L., Vahling-Armstrong, C. M., Chastagner, G. A., and Schroeder, B. K. 2014. Host ranges of North American isolates of *Penicillium* causing blue mold of bulb crops. Crop Prot. 64:129-136.

El Halouat, A., and Debevere, J. M. 1997. Effect of water activity, modified atmosphere packaging and storage temperature on spore germination of moulds isolated from prunes. Int. J. Food Microbiol. 35:41-48.

Gálvez, L., Urbaniak, M., Waśkiewicz, A., Stępień, Ł., and Palmero, D. 2017. *Fusarium proliferatum* – Causal agent of garlic bulb rot in Spain: Genetic variability and mycotoxin production. Food Microbiol. 67:41-48.

Górna, K., Pawłowicz, I., Waśkiewicz, A., and Stepień, Ł. 2016. *Fusarium proliferatum* strains change fumonisin biosynthesis and accumulation when exposed to host plant extracts. Fungal Biol. 120:884-893.

Jurado, M., Marín, P., Callejas, C., Moretti, A., Vázquez, C., and González-Jaén, M. T. 2010. Genetic variability and fumonisin production by *Fusarium proliferatum*. Food Microbiol. 27:50-57.

Kenényi, Z., Mulé, G., Moretti, A., Waalwijk, C., and Hornok, L. 2002. Fertility and mating type assessment within *Fusarium proliferatum* isolates from different host plants. Pages 55-68 in: *Fusarium* – Mycotoxins, Taxonomy and Pathogenicity. Proceedings of the Seventh International Symposium, September 4-7 (Vol. 43A). P. Chelkowski, P. Golínski, M. Kozlowska, H. Kwasna, and J. Perkowski, eds. Warsaw Agricultural University, Poznán, Poland.

Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, IA.

Leyronas, C., Chrétien, P. L., Troulet, C., Duffaud, M., Villeneuve, F., Morris, C. E., and Hunyadi, H. 2018. First report of *Fusarium proliferatum* causing garlic clove rot in France. Plant Dis. 102:2658.

Lopez-Bellido, F., Lopez-Bellido, R., Muñoz-Romero, V., Fernandez-Garcia, P., and Lopez-Bellido, L. 2016. New phenological growth stages of garlic (*Allium sativum*). Ann. Appl. Biol. 169:423-439.

Marín, S., Sanchis, V., Teixido, A., Saenz, R., Ramos, A. J., Vinas, I., and Magan, N. 1996. Water and temperature relations and microconidial germination of *Fusarium moniliforme* and *Fusarium proliferatum* from maize. Can. J. Microbiol. 42:1045-1050.

Matuo, T., Miyagawa, M., and Saito, H. 1986. *Fusarium oxysporum* f. sp. *garlic* n. f. sp. causing basal rot of garlic. Ann. Phytopathol. Soc. Jpn. 52: 860-864.

Mbofung, G. C. Y., and Pryor, B. M. 2010. A PCR-based assay for detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed. Plant Dis. 94: 860-866.

Moharam, M. H. A., Farrag, E. S. H., and Mohamed, M. D. A. 2013. Pathogenic fungi in garlic seed cloves and first report of *Fusarium proliferatum* causing cloves rot of stored bulbs in upper Egypt. Arch. Phytopathol. Plant Prot. 46: 2096-2103.

Nicolaisen, M., Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., Justesen, A. F., and Justesen, A. F. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. J. Microbiol. Methods 76: 234-240.

Owens, P. R., and Rutledge, E. M. 2005. Morphology. Pages 511-520 in: Encyclopaedia of Soils in the Environment. D. Hillel, ed. Elsevier, Oxford, U.K.

Palmero, D., De Cara, M., Iglesias, C., Moreno, M. M., González, N., and Tello, J. C. 2010. First report of *Fusarium proliferatum* causing rot of garlic bulbs in Spain. Plant Dis. 94:277.

Palmero, D., De Cara, M., Nosir, W., Gàlvez, L., Cruz, A., Woodward, S., and Tello, J. C. 2012. *Fusarium proliferatum* isolated from garlic in Spain: Identification, toxigenic potential and pathogenicity on related *Allium* species. Phytopathol. Mediterr. 51:207-218.

Proctor, R. H., Desjardins, A. E., Moretti, A., Gullino, R. N., and Strange, M. L. 2010. Biological and chemical complexity of *Fusarium proliferatum*. Pages 97-111 in: The Role of Plant Pathology in Food Safety and Food Security. Springer, Dordrecht, the Netherlands.

Quesada-Ocampo, L. M., Butler, S., Withers, S., and Ivors, K. 2014. First report of *Fusarium* rot of garlic bulbs caused by *Fusarium proliferatum* in North Carolina. Plant Dis. 98:1009.

Regione Emilia-Romagna. 2019. Disciplinare di produzione integrata della regione Emilia Romagna. Retrieved from https://agricoltura.regione.emilia-romagna.it/produzioni-agroalimentari/temi/bio-agro-climambiente/agricoltura-integrata/disciplinari-produzione-integrata-vegetale/Collezione-dpi/dpi_2020/orticole-2020.

Rengwalska, M. M., and Simon, P. W. 1986. Laboratory evaluation of pink root and Fusarium basal rot resistance in garlic. Plant Dis. 70:670-672.

Rheeder, J. P., Marasas, W. F. O., and Vismer, H. F. 2002. Production of fumonisin analogs by *Fusarium* species. Appl. Environ. Microbiol. 68: 2101-2105.

Salvalaggio, A. E., and Ridao, A. 2013. First report of *Fusarium proliferatum* causing rot on garlic and onion in Argentina. Plant Dis. 97:556.

Sankar, N. R., and Babu, G. P. 2012. First report of *Fusarium proliferatum* causing rot of garlic bulbs (*Allium sativum*) in India. Plant Dis. 96:290.

Schwartz, H. F., and Mohan, S. K. 2016. PART I: Infectious/biotic diseases. Pages 8-86 in: Compendium of Onion and Garlic Diseases and Pests, 2nd Ed. H. F. Schwartz and S. K. Mohan, eds. American Phytopathological Society, St. Paul, MN.

Seefelder, W., Gossmann, M., and Humpf, H.-U. 2002. Analysis of fumonisin B_1 in *Fusarium proliferatum*-infected asparagus spears and garlic bulbs from Germany by liquid chromatography–electrospray ionization mass spectrometry. J. Agric. Food Chem. 50:2778-2781.

Sharma, R. C., and Sharma, J. N., eds. 2005. Integrated Plant Disease Management. Scientific Publishers, Jodhpur, India.

Spagnoli, S. 2014. La qualità carta vincente contro l'import a basso prezzo. Agricoltura. 6:36-37.

Stankovic, S., Levic, J., Petrovic, T., Logrieco, A., and Moretti, A. 2007. Pathogenicity and mycotoxin production by *Fusarium proliferatum* isolated from onion and garlic in Serbia. Eur. J. Plant Pathol. 118:165-172.

Stępień, Ł., Koczyk, G., and Waśkiewicz, A. 2011. Genetic and phenotypic variation of *Fusarium proliferatum* isolates from different host species. J. Appl. Genet. 52:487-496.

Stępień, Ł., Waśkiewicz, A., and Wilman, K. 2015. Host extract modulates metabolism and fumonisin biosynthesis by the plant-pathogenic fungus *Fusarium proliferatum*. Int. J. Food Microbiol. 193:74-81.

Tonti, S., Prà, M. D., Nipoti, P., Prodi, A., and Alberti, I. 2012. First report of *Fusarium proliferatum* causing rot of stored garlic bulbs (*Allium sativum* L.) in Italy. J. Phytopathol. 160:761-763.