

Research Article

The Abundance and Diversity of Soil Fungi in Continuously Monocropped Chrysanthemum

Aiping Song, Shuang Zhao, Sisi Chen, Jiafu Jiang, Sumei Chen, Huiyun Li, Yu Chen, Xi Chen, Weimin Fang, and Fadi Chen

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

Correspondence should be addressed to Weimin Fang; fangwm@njau.edu.cn and Fadi Chen; chenfd@njau.edu.cn

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Chrysanthemum is an important ornamental plant which is increasingly being monocropped. Monocropping is known to affect both fungal abundance and species diversity. Here, quantitative PCR allied with DGGE analysis was used to show that fungi were more abundant in the rhizosphere than in the bulk soil and that the fungal populations changed during the growth cycle of the chrysanthemum. The majority of amplified fragments appeared to derive from *Fusarium* species, and *F. oxysporum* and *F. solani* proved to be the major pathogenic species which are built up by monocropping.

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium*) is an important ornamental species, particularly in China. With the increasingly urbanized Chinese population experiencing a marked rise in its standard of living, chrysanthemum production is rising. As is also the case for many crops [1–5], long-term monocropping of chrysanthemum depresses crop productivity, in terms of both quantity and quality. One of the major causes for this decline in productivity is thought to be the changed nature of the soil fungal population and specifically the buildup of soil-borne pathogens. Soil fungi are important in the context of nutrient cycling and transport and carbon recycling [6–8]. Although some fungal species are pathogenic to plants, there are also those which are pathogenic to insect pests and so are clearly beneficial [9]. Only a small proportion of the full spectrum of soil fungi species is readily isolatable using *in vitro* culture, but species identification and quantification methods based on the polymerase chain reaction (PCR) have made a considerable impact in this field. In particular, the combination of a PCR-based assay of the variable 18S rRNA gene and amplicon separation using denaturing gradient gel electrophoresis (DGGE) has been used to derive a much more complete picture of the soil fungal community than what has been achievable in the past [10].

The physical and chemical environment in the rhizosphere is heavily influenced by the living root [11–13], which also affects the local abundance and diversity of soil microbes [14]. Soil-borne pathogens are attracted to the roots of their host species via their perception of specific molecules secreted by the plant into the soil [15, 16]. In a monocropping situation, it is this mechanism which is largely responsible for the buildup of pathogen inoculum over time.

As yet there has been little research focus on the soil microbial community associated with ornamental species. The dynamics of the bacterial component of the chrysanthemum soil microflora were described in some detail by Duineveld et al. [17, 18], but no published literature relates to the fungal component of the soil microflora. Here, we have investigated fungal abundance and diversity in soil supporting the growth of chrysanthemum using real time PCR and DGGE. The aims were to assess whether fungal abundance and diversity were affected by the growth stage of the plant and/or by continuous monocropping and to identify which fungal species are responsible for productivity decline in monocropped chrysanthemum.

2. Materials and Methods

2.1. Soil and Plant Growth. Soil used for three years of continuously monocropped chrysanthemum was obtained from

continuously monocropped to chrysanthemum for three years, and infecting fungi were recovered from diseased root, stem, and rhizome material using the conventional organizational separation [30, 31]. The identification of fungal species was carried out by applying both DNA diagnostics and morphological characterization. The former involved the PCR amplification and sequencing of the nuclear ribosomal repeat unit internal transcribed spacer (ITS) sequence, based on the ITS1F and ITS4 primers (Table 1) [24, 25]. The ITS sequences of isolated strains were scanned by BLAST [27] against the GenBank nucleotide sequence database. The morphological characterization involved the front and back cultures characters of isolates cultured on potato dextrose agar (PDA) and the form of the macro- and microconidia [32].

2.8. Pathogen Bioassay. Spore suspensions of putative pathogens were obtained from 14-day-old cultures on PDA by adding sterile water to the surface of the Petri dish. The suspension was subsequently filtered through four layers of cheesecloth [2], and the spore concentration was adjusted to 1×10^7 CFU per mL using a haemocytometer. Plants were inoculated and scored after 28 days, following the methods given by Huang et al. [33].

3. Results

3.1. Abundance of Soil Fungi. As estimated from the output of the real time PCR, the number of fungal colony-forming units per gram of rhizosphere soil (cfu g^{-1}) was 2.20×10^8 at the seedling stage, 1.97×10^8 at the vegetative stage, and 2.26×10^8 at the reproductive stage; these levels of abundance were all significantly higher than what was present in the bulk soil (resp., 0.29, 0.34, and 0.58×10^8 cfu g^{-1}) (Figure 1). The DNA extracted from every soil sample tested positive when amplified using the fungal 18S rDNA primers Fung-GC and NS1, and 28 amplified fragments were taken forward for sequencing (Figure 2). The BLAST results associated with some of these, as detailed in Table 2, implied that a number of common plant pathogens were well represented: these included *Magnaporthe grisea* (rice blast), *Rhizoctonia solani* (wide host range), and the two *Fusarium* spp., *F. oxysporum* and *F. solani*. The latter two species were particularly well represented in the rhizosphere during the seedling and reproductive stages depending on the DGGE bands' profiles, while the presence of *R. solani* was detected at all three stages of chrysanthemum development, but at a lower intensity. Evidence of the presence of beneficial fungi in the rhizosphere was provided by the amplification of product from *Chaetomium globosum* (Band 12–6) (Figure 2). The DGGE profiles indicated that the complexity and abundance of soil fungi was greater in the rhizosphere samples than in the bulk soil (Figure 2). A comparison between the two profiles suggested a level of similarity of 59% based on the UPGMA algorithm, and the recovery of the same fragment from duplicate samples showed that the DNA isolation, PCR, and electrophoretic procedures had all been reliable (Figure 3). Overall, *Ascomycete* species were the most abundant (68% of all identified species, Table 2), followed by *Basidiomycetes* (21%).

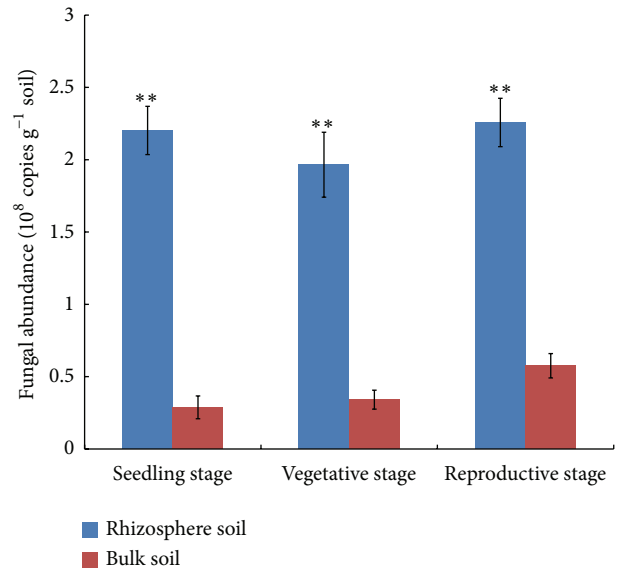


FIGURE 1: The abundance of fungi in the rhizosphere and bulk soil, as indicated by a real time PCR estimation of the copy number of an 18S rDNA fragment. Standard error bars calculated from three replicates. Significant differences based on Student's *t*-test indicated by asterisks (** $P < 0.01$).

3.2. Analysis of Fungal Diversity. Our results demonstrated that the fungi diversity in the rhizosphere soil was different from that in the bulk soil (Table 3). In the rhizosphere soil sampled from plants at the vegetative stage, S (35), H' (3.48), and $1/D$ (29.53) were greater than in the bulk soil sampled from plants at the same stage ($S = 20$, $H' = 2.85$, $1/D = 14.75$). The value of S in the bulk soil was also lower than that in the rhizosphere soil at the seedling stage, but during the reproductive stage, S was higher in the bulk soil. However, the E parameter remained relatively constant throughout, lying in the range of 0.95–0.99.

3.3. The Isolation and Bioassay of Pathogens Isolated from Diseased Chrysanthemum. After five days of *in vitro* culture, 15 fungal strains were isolated from various diseased plant tissues. On the basis of their ITS sequences, it was possible to identify that 11 of these 15 isolates shared 97% similarity with *F. solani* and the other four shared 98% similarity with *F. oxysporum*. One of the putative *F. solani* strains (CFD-1, see Figures 4(a)–4(d)) and one of the putative *F. oxysporum* strains (CFD-1, Figures 4(e)–4(h)) were used for a reinoculation test. The resulting wilt index and infection rate measured 28 days after inoculation (dpi) were 3.6 and 96.3% for *F. solani* CFD-1 and 3.7 and 97.9% for *F. oxysporum* CFD-1 (Table 4). The wilt index following inoculation with *F. solani* CFD-1 was zero at seven dpi, 1.2 at 14 dpi, and 1.9 at 21 dpi, while the time course development of disease following inoculation with *F. oxysporum* CFD-1 was zero at seven dpi, 0.8 at 14 dpi, and 2.1 at 21 dpi. The appearance of the plants as the disease developed is displayed in Figure 5. The pathogen reisolated from the inoculated plants was identical to the one used for the inoculation by ITS sequencing and morphology.

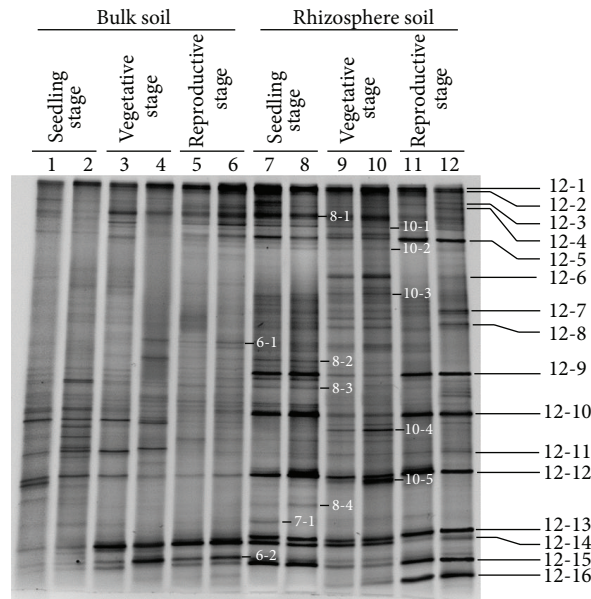


FIGURE 2: DGGE profiles of 18S rDNA fragments present in DNA extracted from bulk soil (lanes 1–6) and rhizosphere (lanes 7–12) sampled at various developmental stages of growing chrysanthemum plants. Lanes 1, 2, 7, and 8: seedling stage, lanes 3, 4, 9, and 10: vegetative stage, and lanes 5, 6, 11, and 12: reproductive stage. Fragments excised for sequencing are indicated by numbers (Table 2).

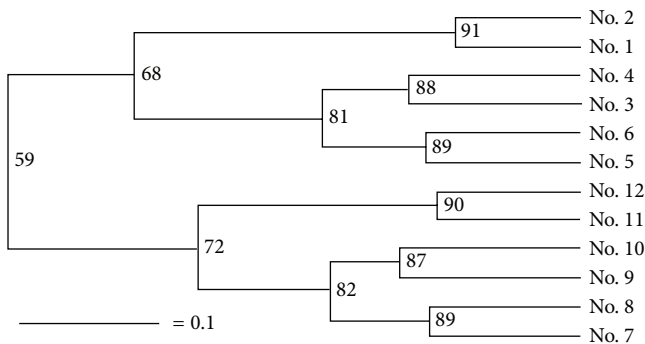


FIGURE 3: Phylogeny of the soil microflora, derived from 18S rDNA DGGE profiles. No. 1–no. 6: bulk soil samples and no. 7–no. 12: rhizosphere samples. No. 1, no. 2, no. 7, and no. 8: soil from plants at the seedling stage, no. 3, no. 4, no. 9, and no. 10: at the vegetative stage, and no. 5, no. 6, no. 11, and no. 12: at the reproductive stage.

4. Discussion

Plants exert a strong influence on the structure and turnover of the rhizosphere fungal community [34–36]. There was little evidence from the current experiments that the abundance of fungi, either in the rhizosphere or in the bulk soil, was responsive to the developmental stage of the chrysanthemum plant (Figure 1). This lack of response may be related to the way in which the soil microflora had been influenced by continuous monocropping. Fungi were more abundant in the rhizosphere than in the bulk soil, presumably because carbohydrate-based exudates from the plant root encouraged the development of a localized higher microbial population size [13, 36, 37].

It has been recognized that a molecular marker-based method of characterizing the components of a complex population can be affected by biases arising from any one of the DNA extraction protocol, the choice of primers, and differential PCR amplifiability [38]. However, it has been demonstrated that a reduced number of PCR cycles and mixing replicate reactions do reduce the risk of bias [39, 40], and this was therefore the approach adopted here to maximize the probability that any differences identified were not experimental artefacts.

The diversity of the DGGE profiles and the variation in the relative abundance of specific amplicons showed that rhizosphere is a significant driver of the structure of the soil microflora community. Furthermore, the plant development stage also influenced fungi diversity significantly, a result which is inconsistent with the claim that the plant only has a minor influence on the constitution of the rhizosphere fungal community [20, 41]. The reason for this inconsistency was likely that the different soil types and sampling methods lead to the different results.

The incidence of wilting in continuously monocropped chrysanthemum crops is most frequent at the seedling stage, followed by during the reproductive stage, but only occurs rarely during the vegetative stage (data not shown). The generally held belief is that this wilting is the consequence of the buildup of soil-borne pathogens over the previous cropping cycle(s) [2, 4]. The present investigation suggested a potential explanation. The abundance of *F. oxysporum* and *F. solani* was at its peak during the seedling stage, while during the vegetative stage it decreased at the same time as the abundance of beneficial fungi increased (Figure 2). If, as has been suggested by Yu and Matsui [42], the constitution of root exudates is developmentally regulated, then the expectation is that

TABLE 2: Most closely related sequences to those derived from selected 18S rDNA amplicons separated by DGGE.

Band(s)	Most closely related bacterial sequence	Identity (%)	Accession no.
12-1	<i>Magnaporthe grisea</i> strain Guy 11	98%	AF277123.1
12-2	Uncultured <i>Cyathus</i> clone F3	98%	EF640307.1
12-3	<i>Trichoderma koningiopsis</i> strain T-440	100%	JQ278020.1
12-4	<i>Coniochaeta ligniaria</i>	99%	AY198389.1
8-1	<i>Fusarium</i> sp. EF1	100%	GQ166777.1
10-1	<i>Myceliophthora hinnulea</i> strain ATCC 52474	100%	JQ067909.1
12-5	<i>Cordyceps sinensis</i>	99%	AB067700.1
10-2	<i>Rhizoctonia solani</i> isolate Q1	99%	JF499071.1
12-6	<i>Chaetomium globosum</i> isolate NK-102	98%	HQ529774.1
10-3	<i>Termitomyces clypeatus</i> isolate TB	98%	HM036344.1
12-7	<i>Bionectria ochroleuca</i> strain WY-1	97%	GU112755.1
12-8	<i>Cyphelium tigillare</i>	100%	AF241545.1
6-1	<i>Trechispora alnicola</i> isolate AFTOL-ID 665	98%	AY657012.1
8-2	<i>Chrysomphalina grossula</i> isolate AFTOL-ID 981	99%	AY752969.1
12-9	<i>Fusarium oxysporum</i> strain SP-2	99%	HM152769.1
8-3	<i>Fusarium</i> sp. MBS1	100%	FJ613599.1
12-10	<i>Fusarium solani</i> strain 421502	98%	EF397944.1
10-4	<i>Aspergillus ustus</i> isolate Li-62	99%	GU573851.1
12-11	<i>Acremonium sclerotigenum</i> strain CBS 124.42	98%	HQ232209.1
12-12	<i>Campanella</i> sp. MCA2235	98%	AY916675.1
10-3	<i>Crinipellis zonata</i> strain OKM 25450	99%	AY916691.1
8-4	Uncultured fungus isolate DGGE gel band 22	95%	JN591717.1
7-1	<i>Hypocrea jecorina</i> strain EIM-30	98%	JN831373.1
12-13	<i>Geomyces destructans</i> isolate MmyotGER-1	100%	GU999983.1
12-14	<i>Pythium cylindrosporium</i> isolate 275	99%	EU199112.1
6-2	<i>Pythium boreale</i> strain CBS 551.88	99%	EF418927.1
12-15	Uncultured soil <i>basidiomycete</i> clone F7	100%	JN656541.1
12-16	<i>Emericellopsis maritima</i> isolate AFTOL-ID 999	98%	FJ176807.1

TABLE 3: Diversity indices associated with the fungal flora present in the rhizosphere and bulk soil samples of continuously monocropped chrysanthemum.

Diversity index	Bulk soil			Rhizosphere soil		
	Seedling stage	Vegetative stage	Reproductive stage	Seedling stage	Vegetative stage	Reproductive stage
S	23	20	30	28	35	26
H'	3.1	2.85	3.26	3.23	3.48	3.15
E	0.99	0.95	0.96	0.97	0.98	0.97
1/D	21.47	14.75	21.27	23.06	29.53	21.44

S: richness, H': Shannon-Wiener diversity index, E: evenness, 1/D: the reciprocal of Simpson's index of diversity.

the fungal community will also vary qualitatively over the course of the plants' development.

The reinoculation test showed that the isolates were indeed pathogenic. This makes it highly likely that the *Fusarium* spp. in question are responsible for the wilt affecting continuously monocropped chrysanthemum. These results may promote the prevention and early diagnosis of *Fusarium* wilt disease, which was prevalent in continuously monocropped chrysanthemum. The abundance of these fungi in the rhizosphere is encouraged by exudates produced by the chrysanthemum root. The present study has established a firm foundation for studying the interaction between

TABLE 4: The pathogenicity of two *Fusarium* sp. isolates present in diseased chrysanthemum plants.

Strains	No. of plants inoculated	Infection rate (%)	Wilt index ^a
<i>Fusarium solani</i> CFD-1	160	96.3	3.6
<i>Fusarium oxysporum</i> CFD-1	144	97.9	3.7

^aRepresenting wilt index at 28 dpi, 0: no wilting; 1: slight wilting on some leaves; 2: most leaves wilted; 3: leaves severely wilted but green; 4: plants wilted and dead.

the chrysanthemum plant and its pathogenic and beneficial rhizosphere fungi.

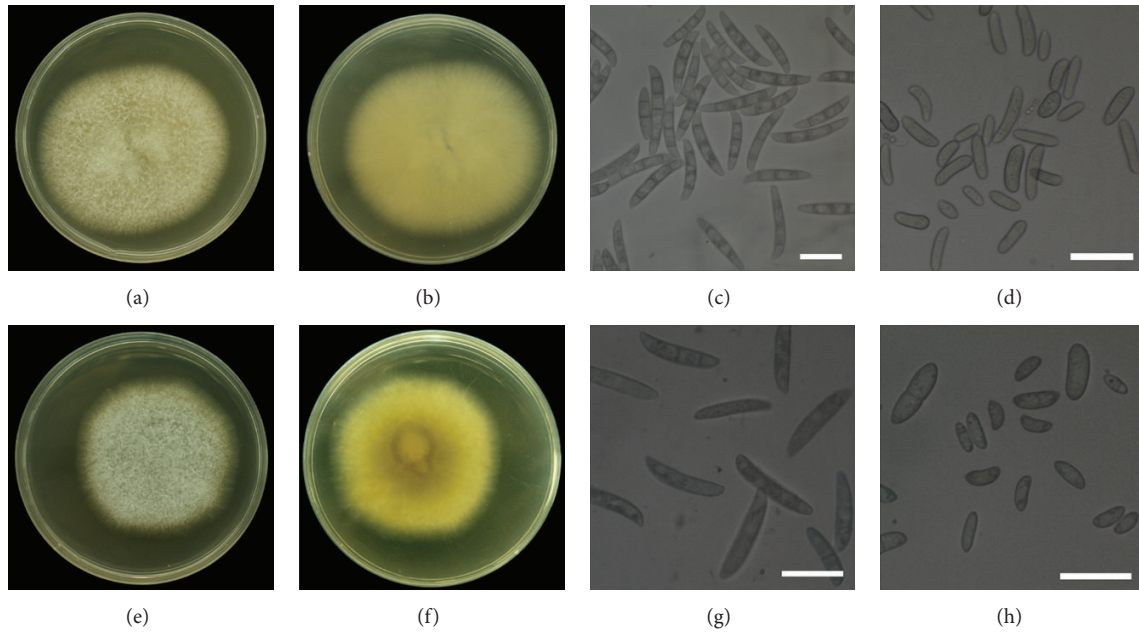


FIGURE 4: Morphology of *F. solani* isolate CFD-1 (a–d) and *F. oxysporum* isolate CFD-1 (e–h). (a, e): front culture character, (b, f): back culture character, (c, g): macroconidia, and (d, h): microconidia. Bars: 50 μ m.

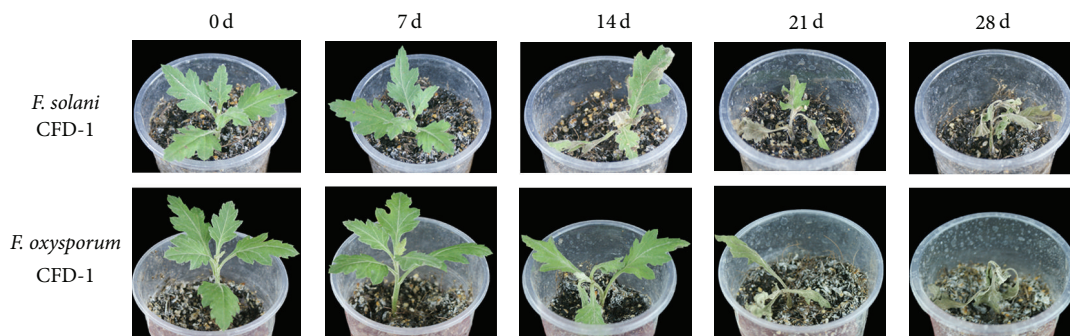


FIGURE 5: The temporal development of disease symptoms in chrysanthemum plants inoculated with either *F. solani* CFD-1 or *F. oxysporum* CFD-1.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Weimin Fang and Fadi Chen equally contributed to this work and should be considered as cocorresponding authors.

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