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Occurrence of *Fusarium* species in asymptomatic barley plants grown in field conditions under differential phosphate regimes

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Occurrence of genus *Fusarium* as endophytes in roots of healthy barley plants cultivated in fields under two levels of phosphorous (P) was examined. *F. avenaceum*, *F. graminearum*, *F. konzum*, *F. oxysporum*, *F. sacchari*, *F. scirpi*, *F. solani* were isolated from asymptomatic surface sterilized root tissues. *F. konzum* and *F. solani* were only present in the roots of fertilized plants, whereas *F. sacchari* was present in unfertilized plants. *F. scirpi* was isolated in highest frequency and relative density, and was higher under P deficiency suggesting that P availability would determinate the occurrence of this species, suggesting the possibility of their association with stressed plants. This is the first report on the occurrence of *Fusarium* spp. as endophytes in healthy barley plants. However, it remains to be determined whether *Fusarium* spp. isolated are nonpathogenic forms and beneficial to barley plants or latent pathogens that could produce disease symptoms in other conditions.

Keywords: Barley (*Hordeum vulgare* L.), endophytic fungi, *Fusarium* species, latent pathogens, nutritional regime.

FUSARIUM is a genus of filamentous fungi distributed in soil and associated with plants mainly as pathogens. The species that integrate this genus can range from nine to well over 80 depending on the taxonomy followed[1]. Its members are saprotrophic principally. The pathogenic nature of the fungus is the main concern in agricultural production because of diseases caused on crops such as *Fusarium* head blight, *Fusarium* wilt, etc.[2].

Endophytic microorganisms establish an asymptomatic infection in the internal tissue of plants, and the colonization is demonstrated by isolation from superficially disinfected tissue, among others[3]. In a functional sense, endophytic micro-organisms grow out of surface-sterilized apparently healthy plant organs when cultured in appropriate media[4]. Petrini[5] proposed to extend the term 'endophyte' to account for those latent pathogens that can live asymptomatically in their hosts for some time in their life. This endophyte–host interaction is an equilibrated antagonism in which the asymptomatic state depends on a balance between fungal virulence and plant defences[6], and the environmental conditions[7], i.e. the disease triangle. Although the *Fusarium* genus is not primarily recognized for establishing endophytic colonization in plants, under this definition it could be considered 'opportunistic' endophytes. Indeed, there are evidences documenting such infections in both wild and cultivated plants[4,8,9].

Barley (*Hordeum vulgare* L.) is an important food grain for both animals and humans, and it is currently the fourth most grown cereal worldwide after wheat, corn and rice. In Argentina, malting barley farming has been evolving to be an attractive commercial option in the international market and benefits the soil structure for rotation with other crops[10] (http://www.minagri.gob.ar/new/0-0/programas/dma/granos/Informe-de-cebada.pdf). In recent years, Argentina has established itself as the main exporter of barley and malt for beer production in South America[11]. However, one of the problems in malting barley marketing is the possible contamination of grains with fungi especially *Fusarium* species that cause outbreaks of gushing[12].

In search of fungal root endophytes from healthy barley plants, typical morphotypes of species belonging to the genus *Fusarium* were found. Therefore, the aim of this study was to analyse the occurrence, frequency and species relative diversity of this genus in barley plants cultivated in the field to evaluate *Fusarium* spp. colonization as influenced by phosphorus nutrition.

During September 2010, eight composite roots/soil samples were collected from healthy commercial barley (*Hordeum vulgare* L. cv. Scarlett) crops located in the Pampas region (Junín, Buenos Aires, Argentina; 34°38′S, 60°56′W). Before raising the crop the soil pH, the moisture level of the soil at different depths (0–20, 20–40 and 40–60 cm), total carbon (Ct), organic matter (OM), nitrates (0–20, 20–40 and 40–60 cm) and phosphorus (0–20 cm) contents were determined, and nitrogen available to plants ($N–NO_3^-$) was derived (Table 1). The crop was grown under two levels of phosphorus (unfertilized and fertilized with 30 kg ha^{-1} of P_2O_5), in plots (4 × 12 m size) laid out in randomized blocks with four replications per treatment. In each sampling block, five subsamples separated from each other by 3–5 m, and at 5 cm from barley plants were collected. A sub-sample was a cylindrical core (2.5 cm diameter and 15 cm deep) of soil containing roots of barley plants which was removed with a hand soil borer. The samples were placed in plastic bags for transport to the laboratory, and stored at 4°C until processed (approximately 5 days after collection). Subsamples from each block were mixed to obtain the composite roots/soil samples. Percentage of mycorrhizal

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Table 1. Chemical analysis of soil

DPT (cm)	pH	Ct (%)	OM (%)	P (ppm)	NO_3^- (ppm)	MSTR (%)	$N–NO_3^-$ (kg/ha)
0–20	5.9	1.06	1.83	17.7	26	23	15
20–40					25	18	14
40–60					17	15	10
Total							39

DPT, Depth; Ct, Total carbon; OM, Organic matter content; P, Phosphorus content; NO_3^-: Nitrate content; MSTR, Moisture level of the soil; $N–NO_3^-$, Nitrogen available to plants.

root colonization was estimated according to McGonigle *et al.*[13].

To ensure that roots were free of lesions, stereomicroscopic and visual inspections were first performed. The isolate of endophytic *Fusarium* species, healthy roots (segments of 2–3 cm) of barley plants were rinsed in running tap water, immersed in ethanol 75% (v/v) for 40 s, followed by sodium hypochlorite 1.5% (v/v) for 7 min and finally rinsed three times with sterile-distilled water. Roots were cut into segments of 5 mm (root tips were discarded because of low fungal colonization observed microscopically) under aseptic conditions and transferred to a petri dish containing 1.5% agar-water (w/v) medium supplemented with chloramphenicol (0.3 mg ml^{-1}) and streptomycin (0.3 mg ml^{-1}) to prevent bacterial growth. Six root segments per petri dish were placed. A total of 48 petri dishes (24 per treatment, 6 per block) were sealed with plastic film to avoid desiccation, incubated at 27°C in the dark and observed periodically. Hyphae emerging from segment tips were sub-cultured onto fresh potato dextrose agar (PDA) for purification of isolates. To ensure that the sterilization method was successful some segments of 2–3 cm were placed in the agar petri dish and incubated at the same conditions with no hyphae appearing.

The isolates with similar colony morphology resembling that of *Fusarium* species were sub-cultured onto standard media used in the identification of *Fusarium* spp., carnation leaf-piece agar (CLA)[14], and PDA tubes. Petri dishes were incubated for 6 to 15 days in a controlled chamber at 25°C under 12 h light/dark conditions. The *Fusarium* species were morphologically identified according to the taxonomic keys proposed by Nelson *et al.*[15], and Leslie and Summerell[1].

To avoid possible mistaken identification at morphological level, a molecular identification from monosporic culture was performed. Representative isolates selected at random were cultured in petri dishes with 2% PDA for 7 days at 25°C under 12 h light/dark conditions. The mycelia were harvested and the genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol[16]. The DNA quality was examined by electrophoresis in 0.8% (w/v) agarose gels containing GelRedTM (Biotium) at 80 V in 1× Trisborate-EDTA buffer for 3 h at room temperature. The DNA was visualized under UV light. DNA concentrations were calculated using a fluorometer (QubitTM-Invitrogen).

Polymerase chain reaction (PCR) analyses (XP Thermal Cycler, BIOER Technology) using available species-specific primers for *F. oxysporum*[17], *F. avenaceum* and *F. equiseti*[18] were made and compared with positive controls. Products from PCR reactions were examined by electrophoresis in 1.5% (w/v) agarose gels containing GelRedTM (Biotium) at 80 V in 1× Trisborate-EDTA buffer for 1 h at room temperature. Fragments were visualized under UV light. The size of DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Genbiotech SRL).

The frequency of isolation (Fr) and relative density (RD) for each *Fusarium* sp. was calculated according to Marasas *et al.*[19]. RD data were subjected to two-way analysis of variance using treatment, species and the possible interaction among these factors as variation source. Means were compared by Tukey's test. This analysis was done using a free version of the INFOSTAT software[20].

A total of 288 root segments obtained from apparently healthy/asymptomatic barley plants were used for isolating possible endophytic fungal species, of which 216 presented hyphal growth, yielding a total of 325 isolates (more than one isolate per root piece). The number of isolates obtained in the treatment without phosphorous (–P) was 184 (56.6%), and 141 (43.4%) fungal isolates were recovered from the fertilized one (+P). Among the 184 isolates collected from blocks without P, 21 belonged to *Fusarium* genus (11.4%). Likewise, 8 isolates of the 141 isolates from P fertilized treatment belonged to the genus *Fusarium* (5.7%) (Table 2). Of the *Fusarium* isolates obtained in this study, seven species of *Fusarium* were identified: *F. avenaceum*, *F. graminearum*, *F. konzum*, *F. oxysporum*, *F. sacchari*, *F. scirpi* and *F. solani*. *F. scirpi* was obtained at the highest frequency in both the treatments (Table 2).

Two-way ANOVA showed a statistically significant interaction between both factors (treatment × species) indicating that the different species were not distributed equally between treatments (Table 3). Post-hoc test indicated that the interaction comes from the fact that the RD of *F. scirpi* between treatments is significantly different (t-test, $P = 0.004$).

Table 2. Frequency and relative density of endophytic *Fusarium* species isolated from root of asymptomatic barley plants

	Incidence of *Fusarium* spp.											
	Block samples						*Fusarium* isolates					
	Number			Frequency (%)*			Number			Relative density (%)**		
	–P	+P	Total	–P	+P	Total	–P	+P	Total	–P	+P	Total
F. avenaceum	2	1	3	50	25	37.5	3	1	4	14.3[a]	12.5[a]	13.8
F. graminearum	1	1	2	25	25	25	3	1	4	14.3[a]	12.5[a]	13.8
F. konzum	0	1	1	0	25	12.5	0	1	1	0[a]	12.5[a]	3.45
F. oxysporum	1	1	2	25	25	25	1	1	2	4.75[a]	12.5[a]	6.9
F. sacchari	1	0	1	25	0	12.5	1	0	1	4.75[a]	0[a]	3.45
F. scirpi	4	2	6	100	50	75	13	2	15	61.9[b]	25[a]	51.7
F. solani	0	1	1	0	25	12.5	0	2	2	0[a]	25[a]	6.9
Total *Fusarium*	4	2	6	100	50	75	21	8	29	11.4***	5.7***	8.9***
Total isolates							184	141	325	56.6***	43.4***	100***

*Frequency (%) = (number of soil samples with occurrence of a *Fusarium* species/total number of samples) × 100; **Relative density (%) = number of isolates of each *Fusarium* spp./Total number of *Fusarium* spp. isolates) × 100; ***Relative density (%) = number of isolates of *Fusarium* spp. or isolates/total number of isolates) × 100. The values displayed in Fr and RD are averages of the 4 blocks. Statistically significant differences are shown with different lowercase letters.

Table 3. Two-way ANOVA preformed from relative density of Table 2

Source of variation	DF	SS	MS	F	P
Treatment	1	714.29	714.29	3.901	0.0549
Species	6	9725.51	1620.92	8.852	<0.0001
Treatment × species	6	6647.73	1107.95	6.050	0.0001
Error	42	7691.16	183.12		
Total	55	24778.68			

DF, Degrees of freedom; SS, sum of squares; MS, mean sum of squares; F, *F*-statistic; P, *P*-value.

Relative density of *F. scirpi* in unfertilized treatment (–P) was significantly higher than RD of any other *Fusarium* spp. isolated from either treatment (Table 2). In contrast, differences in this parameter between the remaining six *Fusarium* spp. isolated were not observed.

The number of *Fusarium* species isolated per soil sample ranged from 0 to 4. All soil samples recollected from unfertilized treatment (–P) showed at least one species of *Fusarium*, and two soil samples collected from P-fertilized did not yield any *Fusarium* spp. (Table 2). In treatment without P, both *F. konzum* and *F. solani* were not present, whereas in the fertilized treatment, *F. sacchari* was the only species not present. The remaining *Fusarium* spp. were isolated from both the fertilized and the unfertilized treatment. *F. scirpi* was present in all soil samples with *Fusarium* species isolated (Table 2).

Endophytic fungi inhabit the inner tissue of living plants without causing any negative effect, and occur in almost all plants on earth[21,22]. This colonization can be demonstrated by isolation from superficially disinfected tissue[3]. In a functional sense, these microorganisms can be cultured on appropriate media[4]. Most endophytes are horizontally transmitted to their hosts, but a few are also vertically transmitted[23,24], and once inside the host, they assume a quiescent or latent state either for an extended period of time or for the whole lifetime of the host plant[22,25].

Petrini[5] proposed to extend the term 'endophyte' to account for those latent pathogens that can live asymptomatically in their hosts for some time in their life. Therefore, when a fungus is isolated as an endophyte it does not exclude the possibility that it can be a weak pathogen or a virulent strain detected during quiescence[6,26].

This concept of endophytism includes infections described by plant pathologists as short endophytic phases or latent infections. Thus, there are several *Fusarium* endophytes with short endophytic phases that become symptomatic pathogenic infections when conditions are appropriate and also *Fusarium* with endophytic nonpathogenic infections that never develop into symptomatic[4,27]. Under this definition, *Fusarium* species isolated in this study could be considered 'opportunistic' endophytes which never become pathogenic under different conditions from here assessed, as was reported earlier[28,29].

The isolation of several species of *Fusarium* from different organs of asymptomatic plants has been reported in various wild and cultivated plant species[4,29–31], but not from barley plants. It is known that *F. oxysporum* has

both pathogenic and non-pathogenic associations with plants[32–36], and is the most studied species that has been frequently isolated from soil and plants[7,19,37–40]. However, this study shows that *F. oxysporum* was one of the least common species together with *F. solani* (RD = 6.9). *F. avenaceum* and *F. graminearum* which are usually found in cereals[1,41–43], recorded a moderate RD value (13.8). On the other hand, only limited information about the presence of *F. sacchari* and *F. konzum* is available in the literature[44–47].

With regard to the occurrence of *Fusarium* species as endophytes, almost exclusive information is available on *F. oxysporum*, where the existence of non-pathogenic strains of this species in different plant species has been shown[31,35,36,48,49]. Recently, the occurrence of *F. solani* and *F. konzum* as potential endophytes has been reported[31,50]. Occurrence of *F. scirpi* isolated in this study from asymptomatic plants, that too with the highest frequency of detection in barley has not been reported earlier (Table 2).

Moreover, the isolation frequency of *F. scirpi* was higher in plants under P deficiency treatment compared to P fertilized ones. Apparently, P availability determines the occurrence of this species. The increment in the frequency of *F. scirpi* as endophyte under P stress conditions (dry mass of barley plants, T-test: –P, 67.6 g DW%; +P, 137.5 g DW; $P < 0.05$)[51] did not follow the normal behaviour of common opportunistic pathogen, in which the development of an endophytic behaviour tends to be associated with plants growing in non-stress conditions[6,7,52]. This differential behaviour encourages further study on *F. scirpi* for examining its possible role in mitigating P stress or other stresses.

On the other hand, the –P treatment not only exhibited a higher level of *F. scirpi* association with barley but also of mycorrhizal colonization, as well (*T*-test: –P, 6.5%; +P, 2.5%; $P < 0.05$). This might partially explain the asymptomatic nature of *F. scirpi* association with barley, as mycorrhizal colonization has been reported to protect plants from pathogenic effects[53–55].

There are reports in which the occurrence of *F. avenaceum* and *F. graminearum* in plants and barley grains is evaluated. However in all cases the plants show symptoms of diseases such as Fusarium head blight[56–59]. In addition, several studies report the occurrence of *F. sacchari* in sugarcane, maize, sorghum and rice plants with disease symptoms, but the occurrence of this species in barley plants, either as an endophyte or as a pathogen, has not been reported[47,60,61].

In summary, this is the first report on the occurrence of species belonging to *Fusarium* genus as endophytes in roots of asymptomatic barley plants cultivated in fields under differential phosphate regimes. However, it remains to be confirmed further, whether these endophytic *Fusarium* spp. isolated in this paper are nonpathogenic forms or latent pathogens. If nonpathogenic, understanding the functional basis of their association with barley plants would clarify their lifestyle as either mutualistic, commensalistic or parasitic in nature for further exploitation to our advantage.

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Agronomic biofortification of zinc in wheat (*Triticum aestivum* L.)

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Zinc malnutrition poses a major health issue for human beings globally. Agronomic bio-fortification explores the feasibility to control the zinc deficiency related disorders of the human population. Field experiment was conducted in a red and lateritic soil of Ranchi on 23 wheat cultivars with soil and foliar applications of $ZnSO_4 \cdot 7H_2O$. Zinc content of wheat grain increased from 38.86 to 77.17 mg/kg with soil application and to 76.49 mg/kg with soil + foliar application of Zn. Total Zn uptake by wheat (grain + straw) cultivars with soil + foliar application of Zn was significantly higher in short (933 g/ha) and long (960 g/ha) duration cultivars compared to that with soil application. Apparent Zn recovery in wheat also improved with soil + foliar application of Zn fertilizer, suggested that agronomic bio-fortification of zinc is possible in wheat and can prevent Zn malnutrition in human beings to a considerable extent.

Keywords: Agronomic, biofortification, *Triticum aestivum* L., red and lateritic soil, zinc deficiency.

ZINC (Zn) deficiency affects more than one-third of the human population in the world[1,2]. Its deficiency in soils of India is widespread[1,3,4] and crops grown in these soils suffer from poor or no yield. A close relationship exists among soils, crops and human health nutrition[5]. According to the World Health Organization[6], about 8 lakh people die annually due to zinc malnutrition, among which more than 50% are children below five years of age. Cereal grains are inherently low both in concentration and bioavailability of Zn, particularly when grown on potentially Zn-deficient soils[7,8]. Release of high-yielding cereal cultivars also contributes to the high incidence of Zn deficiency in human beings by reducing Zn concentration in grain through dilution and in soil through depletion[4]. In most cases, there is an inverse relationship between grain yield and grain Zn concentration[9,10]. Breaking the trade-off between grain yield and grain Zn concentration is an important issue and this can be achieved by breeding, transgenic technology or agronomic approaches[11–14]. Wheat is one of the three major cereal crops (viz. wheat, rice and maize) worldwide and represents the main dietary source of calories, proteins and micronutrients for majority of the world's population, especially in the developing countries[15]. Wheat is responsible for up to 70% of daily calorie intake of the population living in rural regions and is an important source of Zn for human beings living in the developing world[4].

Scanty information is available on regional adaptability of Zn fertilization for biofortification of wheat[16–20]. Field studies have been undertaken to evaluate the acquisition and utilization of zinc by promising wheat cultivars grown under red and lateritic soil condition of India.

Twenty-three cultivars of wheat were selected to study the possibility of agronomic biofortification of zinc under red and lateritic soil condition (Table 1). The cultivars were grouped under two categories, i.e. short (11 cultivars) and long (12 cultivars) maturity duration. Field experiment was conducted during winter (*rabi*) season of 2010–11 at the University Research Farm of Kanke, Ranchi, Jharkhand, India. The experiment was laid out in a strip plot design with three replications. The soil had pH 5.50, electrical conductivity (EC) 0.10 dS/m, organic

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