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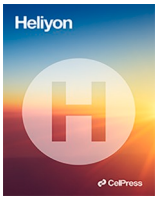

Research article

Endophytic *Alternaria* and *Fusarium* species associated to potato plants (*Solanum tuberosum* L.) in Iran and their capability to produce regulated and emerging mycotoxins

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ABSTRACT

Endophytic fungi live inside virtually every plant species, without causing any apparent disease or damage to the host. Nevertheless, under particular conditions, mutualistic lifestyle of endophytes may change to pathogenic. In this study, the biodiversity of *Alternaria* and *Fusarium* species, the two most abundant endophytic fungi isolated from healthy potato plants in two climatically different regions of Iran, Ardebil in the north-west and Kerman in the south-east, was investigated. Seventy-five *Fusarium* strains and 83 *Alternaria* strains were molecularly characterized by multi-locus gene sequencing. *Alternaria* strains were characterized by the sequences of *gpd* and *caM* gene fragments and the phylogenetic tree was resolved in 3 well-separated clades. Seventy-three strains were included in the clade A, referred as *Alternaria* section, 6 strains were included in clade B, referred as *Ulocladioides* section, and 4 strains were included in clade C, referred as *Infectoriae* section. *Fusarium* strains, identified by sequencing the translation elongation factor 1α (*tef1*), β-tubulin (*tub2*) and internal transcribed spacer (ITS) genomic regions, were assigned to 13 species, *viz. F. brachygibosum*, *F. clavum*, *F. equiseti*, *F. flocciferum*, *F. incarnatum*, *F. nirenbergiae*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. sambucinum*, *F. solani* and *F. thapsinum*. Twenty-six selected strains, representative of *F. equiseti*, *F. nirenbergiae*, *F. oxysporum*, *F. nygamai*, *F. proliferatum*, and *F. sambucinum*, were also tested for production of the mycotoxins deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), T-2 toxin (T-2), beauvericin (BEA), enniatins (ENNs), fumonisins (FBs), fusaric acid (FA) and moniliformin (MON). None of the tested strains produced trichothecene toxins (DON, NIV, DAS and T-2). Two out of 2 *F. equiseti* isolates, 1/6 *F. oxysporum*, 1/3 *F. proliferatum*, and 1/9 *F. nygamai* did not produce any of the tested toxins; the rest of strains produced one or more BEA, ENNs, FBs, FA and MON toxins. The most toxigenic strain, *F. nygamai* ITEM-19012, produced the highest quantities of FBs (7946, 4693 and 4333 μg/g of B1, B2, and B3 respectively), along with the highest quantities of both BEA (4190 μg/g) and MON (538 μg/g). These findings suggest that contamination of potato tubers with mycotoxins in the field or at post-harvest, due to a change in lifestyle of endophytic microflora, should be carefully considered and furtherly investigated.

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1. Introduction

Under natural conditions plants are not individual entities; they are associated with microorganisms to form the plant holobiont [1]. The nature of the interaction of these microorganisms with plants varies from mutualism to parasitism [2]. Endophytic fungi live inside healthy plant tissues without causing any apparent disease symptoms or damage to their hosts. Endophytic fungi have been recovered in all plant tissues, including leaves, stems, roots, flowers and fruits [3], and every plant examined to date was found to harbour at least one species of endophytic fungi. Research on the ecology of endophytic fungi mostly supports their non-pathogenic nature and their capability to enhance biotic and abiotic stress tolerance [4] and improve mineral nutrition in the host plant [5,6], so that endophytes have been proposed as biocontrol agents or beneficial bioinoculants [7]. Nevertheless, conceptual aspects related to the ecological nature of endophytes are still under debate, because some reports have highlighted that, under specific conditions, the mutualistic relationship of endophytes with plants may change to be detrimental [8].

Potato (*Solanum tuberosum* L.) is the fourth-largest world crop, surpassed in total production only by wheat, rice and corn. It is a rich source of carbohydrates for human nutrition and an important part of the diet for over 1.5 billion people worldwide. Iran is the fourth-largest potato producer in Asia, where the crop is extensively cultivated in the provinces of Ardebil, Hamedan, Esfahan, East Azarbaijan, and South Kerman, with the Ardebil province being the most important production area because of its favourable climate conditions [9]. During a large-scale investigation on endophytic fungi of potato plants in two major potato-producing areas of Iran, viz. Ardebil area (northwestern Iran) and South Kerman area (southeastern Iran), almost 400 endophytic fungal strains were isolated from above-ground and below-ground organs of potato plants, and 22 fungal genera, and 52 fungal species were identified [10]. In both the regions investigated, *Alternaria* was the most abundant genus in the above-ground plant parts (stems and leaves) and *Fusarium* in the below-ground plant parts (roots and tubers). Both *Alternaria* and *Fusarium* were previously reported as endophytic in potato. O'Callaghan et al. [11] examined the microbial communities of magainin-producing transgenic lines of *S. tuberosum* in New Zealand, and identified six species of *Fusarium* and two species of *Alternaria*. In Germany, Gotz et al. [12] isolated and identified root endophytic fungi from two different potato lines by traditional techniques and cultivation-independent DNA-based methods. They obtained 67 isolates of *Fusarium* and 73 isolates of *Alternaria*. Marak and Kayang [13] isolated and identified endophytic fungi associated with potato plants from South-West Garo Hills, Meghalaya, India. In that survey, the species *F. oxysporum*, *F. redolens*, *F. semitectum*, *F. solani*, *F. sporotrichioides*, *A. alternata*, *A. brassicicola*, and *A. solani* were identified.

Alternaria species that cause "early blight" and "brown spot" on potato, as well as on tomato, are *A. tomatophila* E.G. Simmons, *A. solani* Sorauer, *A. alternata* (Fr.) Keissl., *A. tenuissima* (Kunze) Wiltshire, *A. infectoria* E.G. Simmons and *A. arborescens* E.G. Simmons [14]. *Alternaria solani* is the second most devastating foliar pathogen of potato crops after *Phytophthora infestans*, worldwide [15]. The common symptoms of *Alternaria* diseases are necrotic lesions on leaves, which are created by the diffusion of fungal toxins [16].

Fusarium dry rot is a postharvest fungal disease affecting potato tubers, which is a cause of up to 60% loss of stored tubers [17]. The disease is caused by several species of *Fusarium*, mainy *F. solani* var. *coeruleum*, *F. sambucinum*, *F. oxysporum*, *F. avenaceum*, and *F. culmorum*. Some *Fusarium* species associated with the disease are known to produce mycotoxins that have been implicated in acute toxicoses of humans and domesticated animals [17,18]. In addition, the prolonged exposure to low sub-lethal doses of mycotoxin may result in chronic toxicoses which often are associated with development of cancer. Several *Fusarium* mycotoxins are regulated in most countries [19]. In addition, concerns about the "emerging mycotoxins" which may co-occur with regulated ones and contribute to the overall health risk of the contaminated commodities, have been growing [19]. *Fusarium graminearum*, a major mycotoxigenic species that produces the regulated mycotoxins zearalenone and tricothecenes in corn and small grains, was reported as a causal agent of *Fusarium* dry rot of potato in USA [20]. It has also been shown that this species is able to produce the trichothecene mycotoxins deoxynivalenon (DON) and nivalenol (NIV) in rotten potato tuber tissue [21]. Potato tubers artificially infected with *F. sambucinum*, another toxigenic species, contained the trichothecene toxin diacetoxyscirpenol (DAS) in concentrations up to 200 μg/tuber [22]. Also, the emerging mycotoxins enniatins were found in potato tuber tissue infected by a complex of six *Fusarium* strains from different sources [23].

In this article, we present the molecular characterization of endophytic strains of *Alternaria* spp. and *Fusarium* spp., isolated from leaf, stem, root, and tuber of potato plants from two geographically diverse potato-producing areas in Iran. We also investigated the mycotoxigenicity of the *Fusarium* strains isolated from tubers and roots, to assess their potential risk of mycotoxin production.

2. Materials and methods

2.1. Sample collection, isolation, and preservation of the endophytic Alternaria and Fusaria

Eighty mature and disease symptomless potato plants, thirty-five from the Ardebil area (northwestern Iran) and forty-five from the South Kerman area (southeastern Iran) were collected in August 2018 and February 2019. In each inspected field, two potato plants were collected from the opposite sides of each field, and the points of sampling were geolocalized. The visited fields were at least 5 km apart from each other. Samples were kept in paper bags under refrigeration at +4 °C and immediately transferred to the laboratory for further processing. Plant organs were surface sterilizedwithin 72 h from collection, as described by Alijani Mamaghani et al. [10]. Briefly, samples were immersed in 70% (v/v) ethanol for 2 min, then in 5% (w/v) sodium hypochlorite for 5 min, rinsed 3 times in sterile distilled water for at least 5 min, dabbed between sterile tissues and let dry in sterile conditions for at least 15 min. Isolations of endophytic fungi from stems, leaves, roots and tubers were done on antibiotic-supplemented Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) followed by single germinated-spore isolation. The cultures were preserved on sterile filter paper pieces stored at

Table 1
Endophytic *Fusarium* strains used for molecular characterization, and accession numbers for their gene sequences in NCBI GeneBank database. The strains used for analysis of mycotoxigenicity are in bold.

Origin and Strain No.[a]	ITEM No.[b]	Plant part	Species	Gene Bank Accession number		
				tef1	*tub2*	ITS
Ardebil Province						
FU-41	18962	Root	*F. clavum*	OQ419383	OQ419308	OQ404982
FU-1	18964	Root	*F. equiseti*	OQ419350	OQ419275	OQ404949
FU-3	18956	Stem	*F. equiseti*	OQ419372	OQ419297	OQ404971
FU-73	18965	Root	*F. equiseti*	OQ419402	OQ419327	OQ405001
FU-87	**18966**	**Root**	***F. equiseti***	OQ419414	OQ419339	OQ405013
FU-94	**18967**	**Tuber**	***F. equiseti***	OQ419422	OQ419347	OQ405021
FU-96	18968	Root	*F. equiseti*	OQ419423	OQ419348	OQ405022
FU-103	18969	Stem	*F. equiseti*	OQ419352	OQ419277	OQ404951
FU-34	18960	Root	*F. flocciferum*	OQ419376	OQ419301	OQ404975
FU-84	**18972**	**Root**	***F. nirenbergiae***	OQ419411	OQ419336	OQ405010
FU-86	**18973**	**Root**	***F. nirenbergiae***	OQ419413	OQ419338	OQ405012
FU-97	18974	Root	*F. nirenbergiae*	OQ419424	OQ419349	OQ405023
FU-2	**18941**	**Tuber**	***F. oxysporum***	OQ419362	OQ419287	OQ404961
FU-35	**18946**	**Tuber**	***F. oxysporum***	OQ419377	OQ419302	OQ404976
FU-66	18986	Tuber	*F. oxysporum*	OQ419398	OQ419323	OQ404997
FU-70	**18987**	**Root**	***F. oxysporum***	OQ419399	OQ419324	OQ404998
FU-92	**18994**	**Leaf**	***F. proliferatum***	OQ419420	OQ419345	OQ405019
FU-36	18990	Root	*F. redolens*	OQ419378	OQ419303	OQ404977
FU-38	18991	Root	*F. redolens*	OQ419380	OQ419305	OQ404979
FU-40	18992	Root	*F. redolens*	OQ419382	OQ419307	OQ404981
FU-59	18997	Tuber	*F. solani*	OQ419391	OQ419316	OQ404990
FU-60	18998	Tuber	*F. solani*	OQ419393	OQ419318	OQ404992
FU-63	19000	Root	*F. solani*	OQ419396	OQ419321	OQ404995
FU-64	–	Root	*F. solani*	OQ419397	OQ419322	OQ404996
FU-72	–	Root	*F. solani*	OQ419401	OQ419326	OQ405000
FU-85	19003	Stem	*F. solani*	OQ419412	OQ419337	OQ405011
Kerman Province						
FU-14	18957	Tuber	*F. brachygibbosum*	OQ419356	OQ419281	OQ404955
FU-81	18961	Root	*F. brachygibbosum*	OQ419408	OQ419333	OQ405007
FU-30	18970	Root	*F. equiseti*	OQ419373	OQ419298	OQ404972
FU-37	18971	Root	*F. equiseti*	OQ419379	OQ419304	OQ404978
FU-83	18963	Root	*F. incarnatum*	OQ419410	OQ419335	OQ405009
FU-20	18975	Tuber	*F. nirenbergiae*	OQ419363	OQ419288	OQ404962
FU-21	18976	Root	*F. nirenbergiae*	OQ419364	OQ419289	OQ404963
FU-24	18977	Tuber	*F. nirenbergiae*	OQ419367	OQ419292	OQ404966
FU-25	18945	Tuber	*F. nirenbergiae*	OQ419368	OQ419293	OQ404967
FU-29	18978	Tuber	*F. nirenbergiae*	OQ419371	OQ419296	OQ404970
FU-33	18979	Root	*F. nirenbergiae*	OQ419375	OQ419300	OQ404974
FU-78	18980	Root	*F. nirenbergiae*	OQ419405	OQ419330	OQ405004
FU-88	**18981**	**Tuber**	***F. nirenbergiae***	OQ419415	OQ419340	OQ405014
FU-89	**18982**	**Root**	***F. nirenbergiae***	OQ419416	OQ419341	OQ405015
FU-91	**18983**	**Tuber**	***F. nirenbergiae***	OQ419419	OQ419344	OQ405018
FU-4	**18947**	**Tuber**	***F. nygamai***	OQ419381	OQ419306	OQ404980
FU-5	**18948**	**Tuber**	***F. nygamai***	OQ419388	OQ419313	OQ404987
FU-6	18984	Root	*F. nygamai*	OQ419392	OQ419317	OQ404991
FU-10	**18949**	**Root**	***F. nygamai***	OQ419351	OQ419276	OQ404950
FU-11	18985	Tuber	*F. nygamai*	OQ419353	OQ419278	OQ404952
FU-12	**18950**	**Tuber**	***F. nygamai***	OQ419354	OQ419279	OQ404953
FU-15	**18951**	**Tuber**	***F. nygamai***	OQ419357	OQ419282	OQ404956
FU-23	**18952**	**Tuber**	***F. nygamai***	OQ419366	OQ419291	OQ404965
FU-26	18953	Root	*F. nygamai*	OQ419369	OQ419294	OQ404968
FU-27	19005	Tuber	*F. nygamai*	OQ419370	OQ419295	OQ404969
FU-31	19006	Root	*F. nygamai*	OQ419374	OQ419299	OQ404973
FU-43	19007	Root	*F. nygamai*	OQ419385	OQ419310	OQ404984
FU-44	19008	Root	*F. nygamai*	OQ419386	OQ419311	OQ404985
FU-48	19009	Root	*F. nygamai*	OQ419387	OQ419312	OQ404986
FU-54	–	Tuber	*F. nygamai*	OQ419389	OQ419314	OQ404988
FU-71	19011	Root	*F. nygamai*	OQ419400	OQ419325	OQ404999
FU-77	**19012**	**Root**	***F. nygamai***	OQ419404	OQ419329	OQ405003
FU-79	**19013**	**Root**	***F. nygamai***	OQ419406	OQ419331	OQ405005
FU-90	**19014**	**Tuber**	***F. nygamai***	OQ419418	OQ419343	OQ405017
FU-8	**18942**	**Tuber**	***F. oxysporum***	OQ419407	OQ419332	OQ405006
FU-9	**18943**	**Tuber**	***F. oxysporum***	OQ419417	OQ419342	OQ405016
FU-18	18944	Stem	*F. oxysporum*	OQ419360	OQ419285	OQ404959
FU-76	**18988**	**Root**	***F. oxysporum***	OQ419403	OQ419328	OQ405002

(*continued on next page*)

Table 1 (*continued*)

Origin and Strain No.[a]	ITEM No.[b]	Plant part	Species	Gene Bank Accession number		
				tef1	*tub2*	ITS
FU-82	18989	Stem	*F. oxysporum*	OQ419409	OQ419334	OQ405008
FU-13	**18954**	**Tuber**	***F. proliferatum***	OQ419355	OQ419280	OQ404954
FU-17	**18955**	**Tuber**	***F. proliferatum***	OQ419359	OQ419284	OQ404958
FU-62	18993	Tuber	*F. proliferatum*	OQ419395	OQ419320	OQ404994
FU-19	**18958**	**Root**	***F. sambucinum***	OQ419361	OQ419286	OQ404960
FU-16	–	Root	*F. solani*	OQ419358	OQ419283	OQ404957
FU-42	18996	Tuber	*F. solani*	OQ419384	OQ419309	OQ404983
FU-61	–	Root	*F. solani*	OQ419394	OQ419319	OQ404993
FU-93	–	Root	*F. solani*	OQ419421	OQ419346	OQ405020
FU-22	18959	Tuber	*F. thapsinum*	OQ419365	OQ419290	OQ404964
FU-55	19015	Tuber	*Rectifusarium robinianum*	OQ419390	OQ419315	OQ404989

[a] Department of Plant Protection, Mycology Laboratory of the College of Agriculture and Natural Resources, University of Tehran, Iran.
[b] Accession number of the strain in the Agro-Food Microbial Culture Collection of Institute of Sciences of Food Production, CNR, Italy (http://server.ispa.cnr.it/ITEM/Collection/).

−20 °C. Pure cultures of the endophytes were deposited in the Mycology Laboratory of the College of Agriculture and Natural Resources, University of Tehran, Iran; representative isolates that were used for molecular identification and analysis of mycotoxins were also cryopreserved at −80 °C in the Agro-Food Microbial Culture Collection of Institute of Sciences of Food Production, CNR, Italy (http://server.ispa.cnr.it/ITEM/Collection/) under an "ITEM" accession number (Tables 1 and 2).

2.2. Morphological identification of endophytic Fusarium and Alternaria strains

Isolates of endophytes putatively assigned to *Fusarium* and *Alternaria* genera based on conidial morphology, were re-isolated from single spores and transferred on specific media to examine their macroscopic and microscopic features for the identification to the species level. *Fusarium* strains were incubated on PDA at 25 °C for 5–7 days with a 12/12 h day/night photoperiod to examine growth rate, colony features and production of chlamydospores; Carnation Leaf Agar (CLA) and Synthetic Nutrient Agar (SNA) were used for examination of macroconidia, chlamydospores, and phialides, according to Leslie and Summerell [24]. Morphological identification of *Alternaria* strains was carried out according to Simmons [25]. Small plugs of pure isolates were cultured on Potato Carrot Agar -PCA [26] and kept under 8/16 h of fluorescent light/dark cycle at 22 °C for 5–7 days. Then, colony features, sporulation patterns, conidial chains, shape, size and septa of conidia and primary and secondary conidiophores were examined.

2.3. Molecular characterization of Fusarium and Alternaria endophytic strains

Seventy-five (26 from Ardebil and 49 from Kerman) *Fusarium* strains (Table 1) and 83 *Alternaria* strains (34 from Ardebil and 49 from Kerman) (Table 2) were selected as representative of the *Fusarium* and *Alternaria* populations from different parts of potato plants, *viz.* leaves, stems, tubers, and roots. Selection of the strains was based on morphological features, sampling location (geographical area and farm), and potato plant tissue type.

For genomic DNA extraction and molecular analyses, the cryopreserved strains at ISPA-CNR were refreshed on PDA and then cultured on cellophane disks overlaid on PDA Petri dishes. After 3 days of growth at 25 °C, mycelia were scraped, transferred to 2 ml microtubes, frozen and lyophilized. Ten to fifteen mg of powdered lyophilized mycelium were used for DNA extraction by using the "Wizard Magnetic DNA Purification System for food" kit (Promega Corporation, Madison, WI), based on the manufacturer's protocol. The quantity and quality of extracted DNA were examined with Thermo-Scientific Nanodrop (LabX, Midland, ON, Canada), and by 0.8 % agarose gel electrophoresis, in comparison with a standard DNA (1 kb DNA Ladder, Fermentas GmbH).

2.3.1. Polymerase chain reaction (PCR) and sequencing

To molecularly identify *Fusarium* and *Alternaria* strains to the species level, and to evaluate the phylogenetic relationships within the two genera, a multi-locus sequencing approach was used. For the *Fusarium* strains, internal transcribed spacer regions (ITS), translation elongation factor 1α (*tef1*) and β-tubulin (*tub2*) genes were selected among the most informative genomic regions. For *Alternaria* strains, glyceraldephyde-3-phosphate dehydrogenase (*gpd*) and calmodulin (*caM*) gene fragments were chosen for the molecular analyses. The sequences of the primers used for PCR and the relevant references are summarised in Table 3.

Each PCR reaction (total volume of 15 μl), containing 15 ng of genomic DNA, 300 nM each primer, 0.8 mM dNTPs, 1x PCR buffer and 0.6 U of Hot Start *Taq* DNA polymerase (Fisher Molecular Biology, Roma, Italy), was performed in the Mastercycler epgradient thermocycler (Eppendorf). The following PCR conditions were used: 95 °C for 2 min; 35 cycles for ITS, *gpd*, and *caM*, and 40 cycles for *tef1* and *tub2* of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s for ITS, 58 °C for 40 s for *tef1*, 58 °C for 30 s for *tub2*, *gpd*, and *caM*, extension step at 72 °C for 50 s, and final extension at 72 °C for 7 min. The quality and quantity of PCR products were examined and visualized by UV light after electrophoresis separation in 1 × TAE buffer, on 1.5 % agarose gel, in comparison with 100 bp DNA ladder (Invitrogen, Thermo Fisher Scientific). The PCR products were purified with enzymatic mixture of Exonuclease I/FastAP thermosensitive alkaline phosphatase (Thermo Fisher Scientific, Vilnius, Lithuania). Subsequent sequencing was performed with the

Table 2
Endophytic *Alternaria* strains used for molecular characterization, and accession numbers for their gene sequences in NCBI GeneBank database.

Origin and Strain No.[a]	Item No.[b]	Plant part	Section	Gene Bank Accession number	
				Gpd	caM
Ardebil Province					
AL-1	19017	Leaf	*Alternaria*	OQ419109	OQ419192
AL-2	19018	Stem	*Alternaria*	OQ419119	OQ419202
AL-3	19019	Stem	*Alternaria*	OQ419129	OQ419212
AL-4	–	Leaf	*Alternaria*	OQ419138	OQ419221
AL-11	–	Stem	*Alternaria*	OQ419111	OQ419194
AL-13	–	Stem	*Alternaria*	OQ419113	OQ419196
AL-15	–	Stem	*Alternaria*	OQ419114	OQ419197
AL-17	19025	Stem	*Alternaria*	OQ419116	OQ419199
AL-18	–	Stem	*Alternaria*	OQ419117	OQ419200
AL-21	–	Stem	*Alternaria*	OQ419120	OQ419203
AL-24	–	Leaf	*Alternaria*	OQ419123	OQ419206
AL-25	–	Stem	*Alternaria*	OQ419124	OQ419207
AL-26	19031	Stem	*Alternaria*	OQ419125	OQ419208
AL-27	–	Leaf	*Alternaria*	OQ419126	OQ419209
AL-28	–	Leaf	*Alternaria*	OQ419127	OQ419210
AL-30	19035	Stem	*Alternaria*	OQ419130	OQ419213
AL-33	–	Stem	*Alternaria*	OQ419132	OQ419215
AL-34	–	Stem	*Alternaria*	OQ419133	OQ419216
AL-37	19040	Leaf	*Alternaria*	OQ419136	OQ419219
AL-57	–	Leaf	*Alternaria*	OQ419157	OQ419240
AL-59	–	Stem	*Alternaria*	OQ419159	OQ419242
AL-67	–	Leaf	*Alternaria*	OQ419162	OQ419245
AL-70	–	Stem	*Alternaria*	OQ419165	OQ419248
AL-73	19033	Stem	*Alternaria*	OQ419168	OQ419251
AL-74	–	Stem	*Alternaria*	OQ419169	OQ419252
AL-80	19037	Stem	*Alternaria*	OQ419175	OQ419258
AL-85	–	Leaf	*Alternaria*	OQ419180	OQ419263
AL-95	19038	Stem	*Alternaria*	OQ419190	OQ419273
AL-96	19039	Leaf	*Alternaria*	OQ419191	OQ419274
AL-16	–	Leaf	*Infectoria*	OQ419115	OQ419198
AL-22	19042	Root	*Infectoria*	OQ419121	OQ419204
AL-23	–	Leaf	*Infectoria*	OQ419122	OQ419205
AL-51	–	Leaf	*Infectoria*	OQ419151	OQ419234
AL-12	19043	Stem	*Ulocladioides*	OQ419112	OQ419195
Kerman Province					
AL-5	–	Leaf	*Alternaria*	OQ419149	OQ419232
AL-10	19022	Leaf	*Alternaria*	OQ419110	OQ419193
AL-19	19027	Stem	*Alternaria*	OQ419118	OQ419201
AL-29	19034	Stem	*Alternaria*	OQ419128	OQ419211
AL-32	19036	Leaf	*Alternaria*	OQ419131	OQ419214
AL-35	–	Leaf	*Alternaria*	OQ419134	OQ419217
AL-36	–	Stem	*Alternaria*	OQ419135	OQ419218
AL-39	–	Leaf	*Alternaria*	OQ419137	OQ419220
AL-40	–	Leaf	*Alternaria*	OQ419139	OQ419222
AL-41	–	Stem	*Alternaria*	OQ419140	OQ419223
AL-42	19017	Leaf	*Alternaria*	OQ419141	OQ419224
AL-43	19020	Leaf	*Alternaria*	OQ419142	OQ419225
AL-44	19021	Leaf	*Alternaria*	OQ419143	OQ419226
AL-45	–	Root	*Alternaria*	OQ419144	OQ419227
AL-46	19023	Stem	*Alternaria*	OQ419145	OQ419228
AL-47	–	Stem	*Alternaria*	OQ419146	OQ419229
AL-50	19024	Leaf	*Alternaria*	OQ419150	OQ419233
AL-52	19026	Leaf	*Alternaria*	OQ419152	OQ419235
AL-53	19028	Leaf	*Alternaria*	OQ419153	OQ419236
AL-55	19029	Stem	*Alternaria*	OQ419155	OQ419238
AL-56	19030	Leaf	*Alternaria*	OQ419156	OQ419239
AL-61	19032	Leaf	*Alternaria*	OQ419160	OQ419243
AL-63	–	Stem	*Alternaria*	OQ419161	OQ419244
AL-68	–	Leaf	*Alternaria*	OQ419163	OQ419246
AL-69	19044	Leaf	*Alternaria*	OQ419164	OQ419247
AL-71	-	Leaf	*Alternaria*	OQ419166	OQ419249
AL-72	19045	Root	*Alternaria*	OQ419167	OQ419250
AL-75	-	Leaf	*Alternaria*	OQ419170	OQ419253
AL-76	-	Leaf	*Alternaria*	OQ419171	OQ419254
AL-77	19046	Leaf	*Alternaria*	OQ419172	OQ419255
AL-78	19047	Leaf	*Alternaria*	OQ419173	OQ419256

(*continued on next page*)

Table 2 (*continued*)

Origin and Strain No.[a]	Item No.[b]	Plant part	Section	Gene Bank Accession number	
				Gpd	caM
AL-79	-	Root	*Alternaria*	OQ419174	OQ419257
AL-81	19048	Leaf	*Alternaria*	OQ419176	OQ419259
AL-82	19049	Stem	*Alternaria*	OQ419177	OQ419260
AL-83	-	Root	*Alternaria*	OQ419178	OQ419261
AL-84	-	Leaf	*Alternaria*	OQ419179	OQ419262
AL-86	-	Leaf	*Alternaria*	OQ419181	OQ419264
AL-87	19050	Leaf	*Alternaria*	OQ419182	OQ419265
AL-88	-	Leaf	*Alternaria*	OQ419183	OQ419266
AL-89	-	Leaf	*Alternaria*	OQ419184	OQ419267
AL-91	19051	Leaf	*Alternaria*	OQ419186	OQ419269
AL-92	19052	Leaf	*Alternaria*	OQ419187	OQ419270
AL-93	-	Leaf	*Alternaria*	OQ419188	OQ419271
AL-94	19053	Leaf	*Alternaria*	OQ419189	OQ419272
AL-48	19054	Stem	*Ulocladioides*	OQ419147	OQ419230
AL-49	19055	Stem	*Ulocladioides*	OQ419148	OQ419231
AL-54	19056	Leaf	*Ulocladioides*	OQ419154	OQ419237
AL-58	–	Stem	*Ulocladioides*	OQ419158	OQ419241
AL-90	19057	Stem	*Ulocladioides*	OQ419185	OQ419268

[a] Department of Plant Protection, Mycology Laboratory of the College of Agriculture and Natural Resources, University of Tehran, Iran.
[b] Accession number of the strain in the Agro-Food Microbial Culture Collection of Institute of Sciences of Food Production, CNR, Italy (http://server.ispa.cnr.it/ITEM/Collection/).

Table 3
Primers used for the molecular analysis of endophytic *Fusarium* spp. and *Alternaria* spp.

Gene	Primer	Sequence (5'-3')	Reference
ITS	ITS5	GGA AGT AAA AGT CGT AAC AAG G	[118]
	ITS4	TCC GCT TAT TGA TAT GC	[118]
tef1	EF1	ATG GGT AAG GAR GAC AAG AC	[119]
	EF2	GGA RGT ACC AGT SAT CAT GTT	[119]
tub2	Bt2a	GGT AAC CAA ATC GGT GCT TTC	[120]
	Bt2b	GGT AAC CAA ATC GGT GCT TTC	[120]
gpd	Gpd1	CAA CGG CTT CGG TCG CAT TG	[121]
	Gpd2	GCC AAG CAG TTG GTT GTG C-	[121]
CaM	CALDF1	AGC AAG TCT CCG AGT TCA AGG	[122]
	CALDR2	CTT CTG CAT CAY CTG GAC G	[122]

Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Each labelled product was purified by filtration through Sephadex G-50 (5%) (Sigma-Aldrich, Saint Louis, MO, USA) and sequenced in "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems, Foster City, CA, USA).

2.3.2. Phylogenetic analysis

The raw DNA sequences were edited, cleaned and assembled using the BioNumerics v. 5.1 software (Applied Maths, Kortrijk, Belgium). For each considered locus, partial FASTA sequences of endophytic *Fusarium* or *Alternaria* strains, and sequences of reference strains, were aligned using the ClustalW algorithm [27]. The sequences of the strains used as reference for *Fusarium* and *Alternaria* specieswere downloaded through the Fusarioid-ID database (http://www.fusarium.org) and the National Center for Biotechnology Information (NCBI).

For each fungal genus, the combined phylogenetic trees were built using the maximum likelihood method with the MEGA software version 7 [28]. The bootstrap analysis [29] was conducted to determine the confidence of internal nodes using a heuristic search with 1000 replicates, removing gaps. In the phylogenetic analyses of *Fusarium*, the strain *Atractium crassum* CBS 180.31 was used as outgroup taxon. Whereas, for the phylogenetic analyses of *Alternaria*, *A. malorum* CBS 135.31, belonging to the Chalastospora section, was utilized. Sequences obtained in this study were deposited in the GenBank database, with the accession numbers listed in Tables 1 and 2.

2.4. Production of mycotoxins by the endophytic fusarium isolates

Twenty-six *Fusarium* isolates belonging to 6 species, *viz. F. equiseti* (2 isolates), *F. nirenbergiae* (5 isolates), *F. oxysporum* (6 isolates), *F. nygamai* (9), *F. proliferatum* (3 strains), and *F. sambucinum* (1 strain), mostly isolated from tubers, were tested for mycotoxin production in duplicate (Table 1). For production of mycotoxins, isolates were grown on 50 g of rice in 250 mL Erlenmeyer Flasks left to imbibe overnight with 30 mL (approx. 60% v/w) distilled water and then sterilized at 121 °C for 30 min. Each flask was inoculated with five pieces of a fresh fungal culture on PDA and incubated for 21 days at 25 °C, shaking every day. Then, the inoculated kernels

were dried at 55 °C for 48 h and finely milled.

2.4.1. Extraction of mycotoxins from rice cultures

Based on the mycotoxigenicity of the *Fusarium* species considered, as reported by Munkvold [30], cultures were analyzed for the presence of beauvericin (BEA), enniatins (ENNs, namely ENN A, ENN A_1, ENN B, ENN B_1), fumonisins B (FBs, namely FB_1, FB_2 and FB_3), fusaric acid (FA), moniliformin (MON), the Type A trichothecenes diacetoxyscirpenol (DAS) and T-2 toxin (T-2), and the type B trichothecenes deoxynivalenol (DON), nivalenol (NIV) and their respective acetylates 3-AcDON, 15-AcDON and 4-AcNIV.

Two different extraction protocols were used. A multi-toxin extraction procedure was used for the extraction of BEA, ENNs, FBs, FA, and MON. One gram of ground rice culture was extracted with 5 mL of methanol/water (70:30, v/v) in an orbital shaker at 250 rpm for 60 min. The extract was filtered using Whatman No. 4 filters (Waters, Milford, MA, USA). One milliliter was diluted with 1 mL of water and then was filtered through 0.20 μm regenerated cellulose filter (Phenomenex, Torrance, CA, USA). The diluted culture extracts were used for the HPLC analyses.

The second procedure was used for the extraction of DON, as described in Quarta et al. [31]. One gram of ground rice culture was extracted in orbital shaker with 5 mL of acetonitrile/water (84:16, v/v) and 1% of acetic acid for 2 h. After filtration through filter paper (Whatman No. 4), 100 μL were diluted with 900 μL ultrapure water. The residue was filtered through 0.20 μm regenerated cellulose filter and analyzed by UHPLC/DAD.

2.4.2. Analyses of beauvericin and enniatins

Analyses of the cyclic hexadepsipeptides BEA and ENNs (ENN A, $ENNA_1$, ENN B, ENN B_1) were carried out by HPLC as described by Prosperini et al. [32] with minor modifications. One hundred microliters of the filtered extract of the culture (first extraction protocol) were injected into an HPLC apparatus (Agilent 1100 Series, Agilent Technology, Santa Clara, CA, USA) equipped with a binary solvent manager, a column heater set at 40 °C and a diode array (DAD) detector that was set at 205 nm wavelength. The analytical column was a Gemini (150 × 4.6 mm, 5 μm; Phenomenex) preceded by a SecurityGuard™ cartridge Gemini (4 × 3 mm, Phenomenex). The mobile phase was water as solvent A and acetonitrile as solvent B, eluted at a flow rate of 1 mL/min. A gradient elution was performed by changing the mobile phase composition. After 5 min at 70% eluent B, the proportion was set at 90% in 10 min, then kept constant for 3 min. The column was re-equilibrated with 70% eluent B for 5min. The retention times were: 11.3 min for BEA; 13.2 min for ENN A; 11.9 min for ENN A_1; 10.5 min for ENN B; 9.1 min for ENN B_1. The detection limits (LOD) based on signal-to-noise ratio of 3:1, were as follows: BEA = 0.01 μg/g, ENN A = 0.2 μg/g, ENN A_1 = 0.5 μg/g, ENN B = 0.06 μg/g, ENN B_1 = 0.07 μg/g. The mycotoxins were quantified by comparing peak areas with the calibration curves obtained with standard solutions.

2.4.3. Analysis of fumonisins

Analyses of FBs (FB_1, FB_2 and FB_3) were carried out by HPLC/FLD according to the procedure described by Haidukowski et al. [33] with minor modifications. The culture extract (first procedure) diluted and filtered (50 μL) was derivatized with o-phtaldialdehyde (50 μL) using the HPLC autosampler Agilent 1100 and injected after 3 min. The analytical column was a SymmetryShield RP18 (15 cm x 4, 6 mm, 5 μm; Waters) set at 30 °C. The mobile phase consisted of a binary gradient applied as follows: the initial composition of the mobile phase 60% of (A) acetonitrile-water-acetic acid (B) acetonitrile-water-acetic acid (60/39/1, v/v/v), was kept constant for 5 min, then B solvent was linearly increased to 88% in 21 min, and kept constant for 4 min. The flow rate of the mobile phase was 1 mL/min. The fluorometric detector was set at wavelengths, ex = 335 nm, em = 440 nm. Retention time was about 16.4 min for FB_1, 25.4 min for FB_2 and 26.6 min for FB_3. Fumonisins were quantified by measuring peak areas and comparing them with the calibration curves obtained with standard solutions. LOD was 0.02 μg/g based on a signal-to-noise ratio of 3:1 for FB_1, FB_2 and FB_3.

2.4.4. Analysis of fusaric acid

FA was determined by HPLC using a 1100 Agilent instrument. The analytical column was a Gemini (150 × 4.6 mm, 5 μm; Phenomenex). The temperature of the column was maintained at 40 °C. Constant flow was set at 1.0 mL/min and mobile phase was 1 % formic acid as solvent A and methanol with 1% of formic acid as solvent B. The starting gradient ratio was 80% of solvent A and the final ratio was set at 30% of solvent A in 15 min, then kept constant for 2 min. FA was detected at 272 nm. Retention time was about 7.9 min, and the LOD was 0.25 μg/g.

2.4.5. Analysis of moniliformin

Analysis of MON was carried out according to the procedure described by Parich et al. [34] with minor modifications. Fifty microliters of the extract obtained with the first procedure were injected into the HPLC apparatus with a column thermostat set at 30 °C and a DAD detector set at 229 nm. The analytical column was a Symmetry C18 (150 × 4.6 mm, 5 μm; Waters). The mobile phase was a mixture of water/formic acid (99:1, v/v, solvent A) and methanol/formic acid (99:1, v/v, solvent B) eluted at a flow rate of 1 mL/min. A gradient elution was performed as follows: 50% B solvent for 5 min; then was linearly increased to 70% in 2 min, 50% at 12 min and kept constant for 5 min. The retention time of mycotoxin was about 3.9 min, and the LOD was 0.1 μg/g.

2.4.6. Analyses of trichothecenes type B (DON, NIV, 3-AcDON and 4-AcNIV)

Analysis of trichothecenes group B was carried out according to the procedure described by Pascale et al. [35]. Seven point 5 μL of extract were injected into a Waters Acquity UPLC/PDA system. The analytical column was Aquity UPLC BEH RP-C18 (2.1 × 100 mm, 1.7 μm; Waters) with an Acquity UPLC column in-line filter (0.2 μm). The column heater was set at 50 °C and the detector at 220 nm. The isocratic flow was set at 0.350 mL/min and the mobile phase was water/methanol (85:15 v/v). Under these analytical conditions

the retention times of target toxins were NIV 1.3 min, DON 2.1 min, 4-AcNIV 4.3 min, 3-AcDON 9.9 min and 15-AcDON 10.5 min. The detection limits (LOD) were 0.02 μg/g for NIV, DON and 4-AcNIV, 0.05 μg/g for 3-AcDON and 15-AcDON. DON production was confirmed using DONTest™ immunoaffinity column (VICAM, Watertown, MA, USA) method. One gram of rice culture was extracted with 5 mL of water in an orbital shaker at 250 rpm for 60 min. The extract was filtered, and 2 mL was passed through immunoaffinity column. After washing with 5 mL of water, DON was eluted with 1.5 mL of methanol. Then, the extract was dried and solubilized with 250 μL of acetonitrile/water (10:90, v/v). Seven point five μL of extract was injected into to UPLC apparatus as described above. The

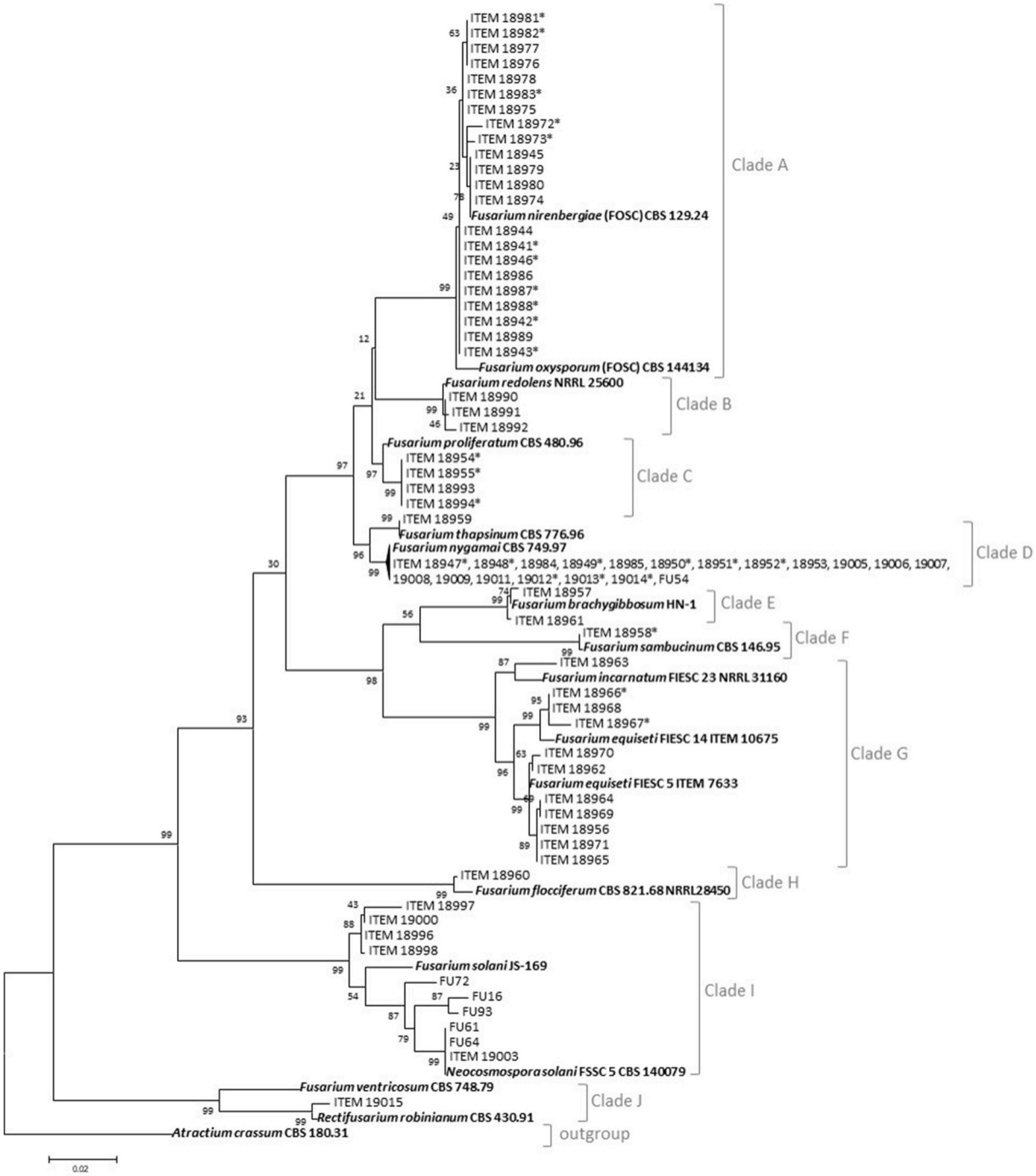

Fig. 1. Phylogenetic tree generated by Maximum Likelihood method (bootstrap 1000 replicates) from combined DNA sequences of *tub*, *tef1*, and ITS fragments of 75 endophytic *Fusarium* strains isolated from different organs of potato plants, in Iran. *Atractium crassum* strain CBS 180.31 was used as an outgroup. Isolates with an asterisk were used for mycotoxin analysis.

detection limit (LOD) of DON based on signal-to-noise ratio of 3:1 was 0.02 μg/g. DON was quantified by comparing peak areas with a calibration curve obtained with standard solutions.

2.4.7. Analyses of trichothecenes type A (DAS and T-2 toxin)

Analysis of trichothecenes group A was carried out according to the procedure described by Pascale et al. [36] with minor modifications. Seven point 5 μL of extract (second protocol) were injected into a Waters Acquity UPLC system. The analytical column was Aquity UPLC BEH RP-C18 (2.1 × 100 mm, 1.7 μm; Waters) with an Acquity UPLC column in-line filter (0.2 μm). The column heater was set at 50 °C and the detector at 202 nm. The chromatographic separation was performed by a gradient elution water as solvent A and acetonitrile as solvent B. The initial composition of the mobile phase. (80 % solvent A, 20% solvent B) was kept constant for 2 min, then solvent B was linearly increased to 50% in 3 min, and kept constant for 1 min. The flow rate of the mobile phase was 0.7 mL/min. Retention times were 1.7 min for DAS and 4.2 min for T-2 toxin. LOD for DAS and T-2 toxin were 4 μg/kg and 8 μg/kg, respectively.

3. Results and discussion

3.1. Endophytic fusarium species

The anamorphic genus *Fusarium* Link is arguably the most agronomically important fungal genus associated to potato plants, since members of the genus are well-known for their phytopathogenicity and mycotoxin production. Out of almost 400 total fungal endophytic isolates from potato, 75 were belonging to the genus *Fusarium*, accounting for 18.75% of total isolates [10]. *Fusaria* were mostly isolated from the underground parts of potato plants (roots and tubers), suggesting some tissue specificity.

Seventy-five isolates were molecularly identified to species level based on the multi-locus sequencing of the genes *tef1*, *tub2* and ITS, and assigned to 13 species, *viz. F. brachygibosum*, *F. clavum*, *F. equiseti*, *F. flocciferum*, *F. incarnatum*, *F. nirenbergiae*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. sambucinum*, *F. solani* and *F. thapsinum* (Table 1). Five *Fusarium* species, namely *F. oxysporum*, *F. redolens*, *F. semitectum*, *F. solani*, and *F. sporotrichioides* were previously reported as endophytic in potato [13]. In our

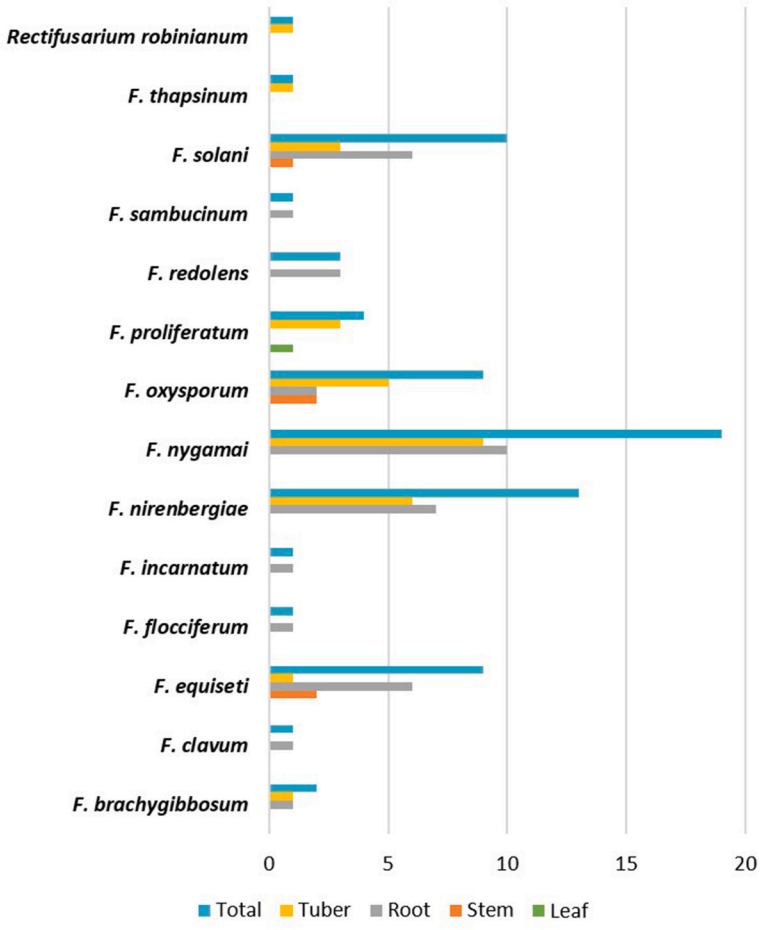

Fig. 2. Occurrence and distribution of endophytic *Fusarium* species in different organs of potato plants.

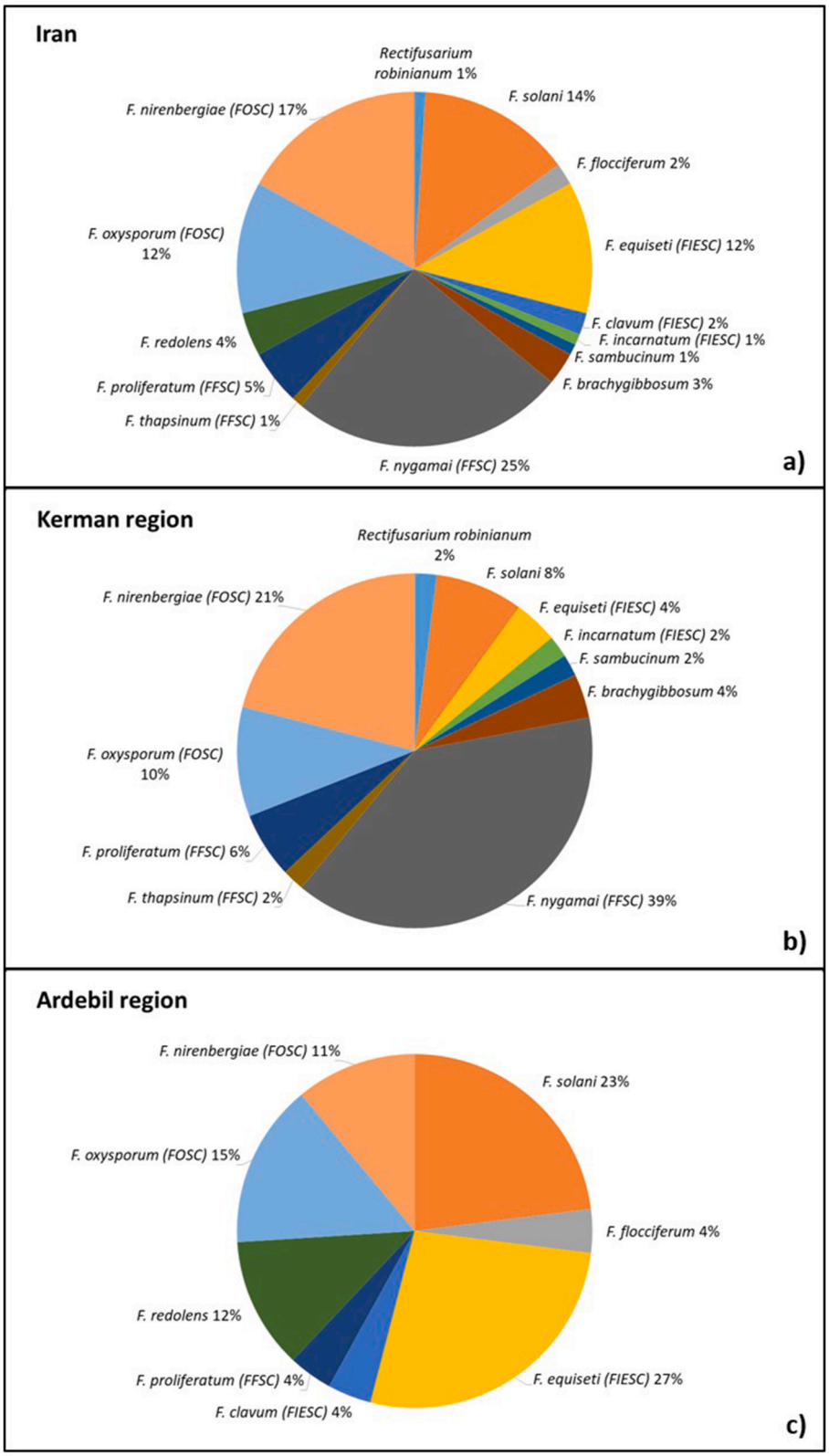

Fig. 3. Frequency of endophytic *Fusarium* species in potato plants from Iran (**a**), detailed for South Kerman (**b**) and Ardebil (**c**) regions. The species complexes are reported in brackets: FFSC: *Fusarium fujikuroi* species complex, FOSC: *F. oxysporum* species complex, FIESC: *F. incarnatum-equiseti* species complex.

survey, we did not find any endophytic strains of either *F. semitectum* or *F. sporotrichioides*.

The phylogenetic analysis of the concatenated sequences of 1337 sites resulted in a phylogenetic combining dataset comprising 92 taxa, including 75 *Fusarium* field strains, 16 *Fusarium* reference sequences and the strain *Atractium crassum* CBS 180.31 as outgroup taxon. Four out of the 79 endophytic *Fusarium* strains isolated (FU-57, -28, −56, −80) were not included in phylogenetic analyses since they did not give PCR products of *tef1*. However, the comparison of *tub* and ITS sequences through the Blast N program (http://www.ncbi.nlm.nih.gov/) allowed us to assign these strains to the genus Plectospharella. A high homology (more than 99%) was found with the deposited sequences of *P. cucumerina* (syn. *Fusarium tabacinum*) strains.

The phylogenetic tree, rooted to outgroup taxon, was resolved in 10 well-separated clades, supported by high significance (bootstrap values more than 96), as reported in Fig. 1. Twenty-two out of 75 strains and *F. oxysporum* CBS 144,134 and *F. nirembergiae* CBS 129.24 reference strains grouped together in a well-supported clade corresponding to the *Fusarium oxysporum* species complex (FOSC, clade A). In this clade, a very low genetic variability was observed. Indeed, all the strains showed high level of similarity among them and with the two reference strains included in the analyses. In particular, the strains ITEM-18945, -18979, −18980, −18974 showed 100% homology with the *F. nirembergiae* reference strain. Clade B, referred to as *Fusarium redolens* species complex (FRSC), grouped the reference strain *F. redolens* NRRL 25600 together with the strains ITEM-18990, -18991, and −18992 with high level of homology. Twenty-four endophytic *Fusarium* strains, identified as belonging to *Fusarium fujikuroi* species complex (FFSC), were grouped in clades C and D. Four *Fusarium* strains, showing 100% of homology among them, clustered with *F. proliferatum* reference strain (clade C). In clade D, two clusters were distinguished: the first cluster contained a single strain (ITEM-18959) that showed high similarity with *F. thapsinum* CBS 776.96 and the second cluster contained majority of the strains (19 out of 20) along with *F. nygamai* CBS 749.97 reference strain.Only two strains (ITEM-18957 and -18961) clustered with *F. brachygibbosum* NH-1 reference strains (clade E) and one single strain with *F. sambucinum* CBS 146.95 strain (Clade F). Eleven endophytic *Fusarium* strains included in the clade G, shared high genetic diversity. This clade included strains assigned to different species of *Fusarium incarnatum-equiseti* species complex (FIESC). One single strain (ITEM-18963) was genetically closely related to the reference strain *F. incarnatum* FIESC 23. Three strains (ITEM-18966, -18968, and −18967) grouped with the reference strains *F. equiseti* FIESC 14. Finally, in a well-supported group, 7 *Fusarium* strains clustered with the reference strain *F. equiseti* FIESC 5. Likewise, large diversity was observed in the clade I, in which 10 endophytic *F. solani* strains clustered with the two *F. solani* strains used as references of the *Fusarium solani* species complex (FSSC). On the other hand, two strains (ITEM-18960 and -19015) showed a high similarity with *F. flocciferum* CBS 821.68, member of *Fusarium tricinctum* species complex, and *F. robinianum* (syn. *Rectifusarium robinianum*), respectively (clades H and J).

Ninety percent of endophytic *Fusaria* strains were isolated from roots and tubers. Only four species, *viz. F. nygamai*, *F. oxysporum* and *F. solani*, were isolated from stems and only one isolate of *F. proliferatum* was obtained from potato leaves (Fig. 2). At a whole, *F. nygamai*, member of FFSC, *F. oxysporum* and *F. nirembergiae*, both members of FOSC, *F. solani* and *F. equiseti* (FIESC), were the most abundant species (Fig. 3a). In particular, *F. nygamai* was predominant in south Kerman (Fig. 3b), and *F. equiseti*, *F. oxysporum* and *F. solani* were more frequently isolated in Ardebil (Fig. 3c). Therefore, *F. nygamai* seems to be more adapted to dry and hot climate conditions, while *F. oxysporum* and *F. solani* can apparently colonize potato plants under diverse climate conditions.

The species *F. brachygibbosum, F. clavum, F. flocciferum* and *F. nirenbergiae* are herein reported as endophytic for the first time. So far, *F. brachygibbosum* has been reported as a plant pathogen of different crops, including wheat [37], maize [38], date palm [39,40], *Cannabis sativa* [41], 2019) and several medicinal plants [42,43], but not potato. *Fusarium clavum* was previously isolated from crop plants cultivated under both conventional and organic farming [44]. The species causes post-harvest contamination of wheat, barely, and maize [45] and associated to leaf spot disease in vegetable plants [46] and leaf wilt in date palm [47]. *Fusarium flocciferum* is a common species in temperate regions, soil, roots, fruits, stems and twigs of various plants [48]. *Fusarium nirenbergiae* is a plant pathogenic species and an agent of vascular diseases such as wilting of common bean [49], passion fruit (Passiflora edulis Sims) [50] and ornamental plants [51], but also reported as the agent of saffron corm rot disease [52].

The species *F. equiseti*, *F. incarnatum*, *F. proliferatum*, *F. nygamai*, *F. sambucinum* and *F. thapsinum* were reported to be endophytes in different plants, but not in potato. Particularly, *F. equiseti* was isolated from leaf of the medicinal plant *Sophora tonkinensis* [53], barley roots [54], and *Salicornia bigelovii* [55]. *Fusarium incarnatum* is the causal agent of postharvest fruit rot in muskmelon (*Cucumis melo*) [56], and of a fruit disease of bell peppers [57], but the species is reportedly endophytic in the mangrove plant *Aegiceras corniculatum* [58]. *Fusarium proliferatum* is a widespread phytopathogen in a number of major crops, including rice, wheat, maize, garlic, asparagus, date palm, and Chinese chive [59], and an endophyte in wheat [60]. On the other hand, few studies report the endophytic associations of *F. nygamai* with medicinal plants, namely *Alhagi graecorum*, *Cressa cretica*, *Citrullus colocynthis*, *Tamarix nilotica*, *Achillea fragrantissima*, *Artemisia sieberi*, and *Neurospora retusa* [42], and rice root [61]. *Fusarium sambucinum*, which is recognized as a major agent of dry rot of potato tubers [24] has also been reported as an endophyte from *Nicotiana tabacum* [62](Zhang et al., 2019). *Fusarium thapsinum* causes stalk rot and grain mold of sorghum [24], but it was also found to be endophytic in the same plant [63].

3.2. Endophytic Alternaria species

The phylogenetic combining dataset comprised 82 taxa, including 83 *Alternaria* field strains, and 9 additional *Alternaria* reference sequences, among which *A. malorum* CBS 135.31 which was used as outgroup taxon. The *Alternaria* isolates were molecularly identified at the species level based on the multi-locus sequencing of the genes *gpd* and *CaM* (Table 3).

The phylogenetic tree, rooted to outgroup taxon, was resolved in 3 well-separated clades, supported by high bootstrap values (Fig. 4). Most of the strains (73 out of 83) were included in the clade A, referred as *Alternaria* section. In this clade, high genetic homology was observed among the strains. Indeed, except for two strains (ITEM-19051 and -19023), 54 *Alternaria* strains clustered with *A. alternata* EGS34-016 and *A. tenuissima* EGS34-015 reference strains; 13 strains were highly similar to *A. lini* CBS 106.34

reference strain, and four strains clustered, in a well-separated group (bootstrap value 84) with *A. arborescens* 39–128 reference strain. In clade B, referred as *Ulocladioides* section, 6 strains grouped with two members of this section, *A. consortialis* and *A. utra*. The strains ITEM-9043, AL-58, and ITEM-19057 were very close to *A. utra* ATCC18040 reference strain; whereas ITEM-19054, -19055 and −19057, showing 100% similarity among them, were very close to *A. consortialis* CBS 201.67 reference strain. Finally, four strains (ITEM-19042, AL-51, -16, and −23) grouped with *A. infectoria* EGS27-193 and *A. viburni* EGS 49–147 reference strains in the *Infectoriae* section (clade C).

Alternaria spp. are ubiquitous fungi that include endophytic, saprophytic, and pathogenic species commonly found in soil, air, food commodities, and on decaying plant tissues [64]. The pathogenic species of *Alternaria* cause major losses on a wide range of crops such as cereals, oil crops, ornamentals, vegetables and fruits [65]. In our survey, out of almost 400 total fungal endophytic isolates, 96 were identified as *Alternaria* spp. [10], that is 24.06 % of total isolates. Ninety-five percent of endophytic *Alternaria* strains were isolated from the above-ground plant parts (stems and leaves). Only five isolates were originated from roots, four of which belonged to the *Alternaria* section and one to the *Infectoriae* section (Fig. 4). It appears that the different climatic conditions in the two Iranian regions of Ardebil and Kerman do not affect significantly the distribution of endophytic *Alternaria*; in both the regions the *Alternaria* section was predominant, supposedly due to greater tolerance to diverse climate conditions.

The species *A. alternata*, *A. tenuissima*, and *A. lini* were the most abundantly occurring species in this study. However, in the last decade *Alternaria* taxonomy has been deeply revised, based on genetic and genomic investigations. Sequencing analyses of the most informative genomic regions led to the conclusion that species that were morphologically different could not be distinguished genetically [66,67]. Therefore, around 35 morpho-species, including *A. alternata*, *A. tenuissima* and *A. lini,* should be considered one single species and synonymized as *A. alternata* [66]. Recently, this finding has been supported also in a genome-wide study based on phylogenomic analyses of *Alternaria* species belonging to *Alternaria* Section [68]. Anyway, morpho-species names continue to be reported in several studies. Previously, only three *Alternaria* species, namely *A. alternata*, *A. solani*, and *A. brassicola* were reported as endophytic in potato [11,13]. *Alternaria alternata* is a cosmopolitan species with a wide host range worldwide, including cereals [69, 70], vegetables [64,71] and halophyte plants [72]. It is pathogenic in many important crops and also lives in asymptomatic symbiosis as an endophyte of many plants [73], including potato, *Nicotiana* spp. [74], forest tree species [75], and *Juncus* spp [76]. This is the first report of *A. lini* as an endophytic species. *Alternaria tenuissima* has been widely reported as endophyte from different plants [77,78], but it is herein reported as an endophyte of potato for the first time. *Alternaria arborescens* was isolated as endophyte from root tissue of *Combretum latifolium* [79], and is also reported as a phytopathogen of potato and tomato [64,71,80,81], and a human pathogen [82]. *Alternaria atra* (previously known as *Ulocladium atra* [65] was reported as an endophyte of common yew (*Taxus baccata* L.) in Iran [83]. *Alternaria consortialis* (previuosly knwon as U. *consortilis* [65], was reported as endophyte of several plants in Iran, including *Prunus* trees [84], and spinach [85], and as a phytopathogen of declined Persian oak trees in Iran [86] and on date palm showing leaf spot in

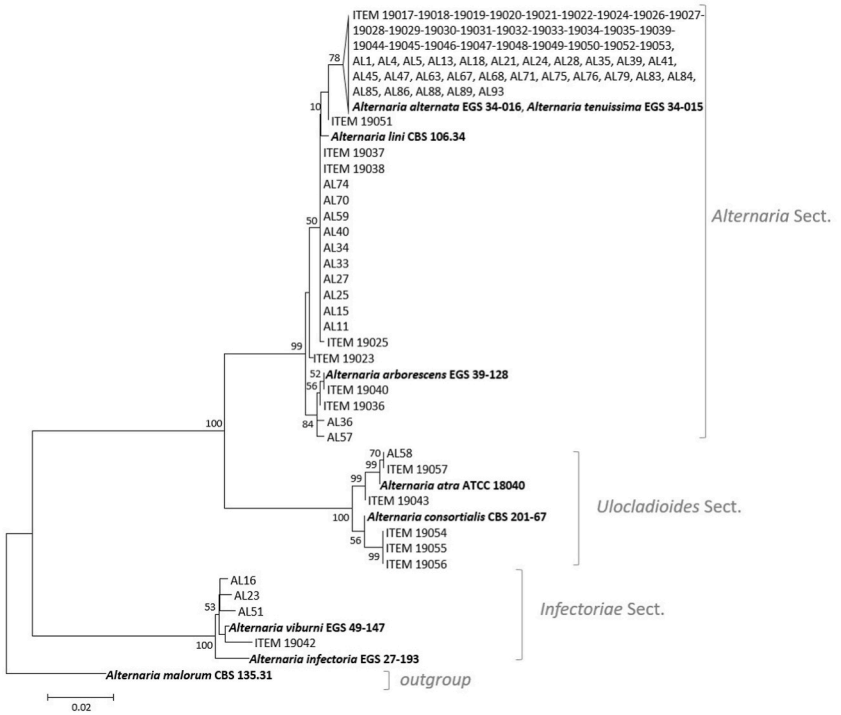

Fig. 4. Phylogenetic tree generated by Maximum Likelihood method (bootstrap 1000 replicates) from combined DNA sequences of *gpd* and *caM* fragments of 83 endophytic *Alternaria* strains isolated from different organs of potato plants, in Iran. *Alternaria malorum* strain CBS 135.31 was used as an the outgroup.

Tunisia [87]. *Alternaria viburni,* previously known as *Lewia viburni* [65], is reported as endophyte from timothy (*Phleum pratense*) and perennial ryegrass [88]. This species belongs to the *Infectoriae* Section, which is phylogenetically divergent from the *Alternaria* Section and characterized by a great genetic variability within and among its species members [67].

While *Alternaria* spp. are mainly regarded as plant pathogenic fungi that cause diseases of aerial parts of plants and are also capable of producing potentially harmful metabolites [89], beneficial effects of endophytic *Alternaria* on plant growth have also been reported [90]. Whether the endophytic *Alternaria* strains associated with potato plants may turn to be pathogenic and under which conditions this may occur, or conversely may have beneficial effect on potato plants, remains to be clarified and is worth of further investigation.

3.3. Mycotoxin production by potato endophytic fusarium strains

The toxigenicity of twenty-six *Fusarium* strains from potato root and tubers was assessed. The strains tested belonged to 6 species, *viz. F. equiseti* (2 strains), *F. nirenbergiae* (5 strains), *F. oxysporum* (6 strains), *F. nygamai* (9 strains), *F. proliferatum* (3 strains), and *F. sambucinum* (1 strain). Twenty-one out of 26 strainswere able to produce one or more mycotoxin (Table 4).

None of the tested strains produced either type A or type B trichothecenes. Two out of two *F. equiseti* strains, 1/6 *F. oxysporum*, 1/3 *F. proliferatum*, and 1/9 *F. nygamai* did not produce any of the other tested toxins. All the 5 strains of *F. nirenbergiae* produced both BEA (from moderate to high quantity, 101.0–1382.5 μg/g) and MON (2.9–82.1 μg/g). *Fusarium oxysporum* showed a more variable mycotoxin profile: ENN A1 was produced by 2/6 strains, BEA by 3/6, MON by 2/6 and FA by 1/6. BEA was found in combination with MON in a single strain; ENN A1 was found in combination with either FA (1 strain) or BEA (1 strain). Among the *F. proliferatum* strains, 1/3 did not produce any of the mycotoxins analyzed, while the other 2 strains produced both BEA and FBs (B1, B2, and B3). In *F. nygamai*, which was the most numerous group, 3/9 strains produced BEA, 4/9 produced FBs (B1, B2, and B3), and 7/9 produced MON. All the fumonisins-producing strains also produced MON and 2 of them produced BEA, as well. One isolate produced BEA in combination with MON. The most toxigenic strain was ITEM-19012, which produced the highest quantities of FBs (7946.5, 4693.0 and 4333.0 μg/g of B1, B2, and B3 respectively), along with the highest quantities of both BEA (4190.0 μg/g) and MON (538.4 μg/g). The only toxin produced by *F. sambucinum* was BEA.

Amongst the regulated mycotoxins, only FBs were produced by potato endophytic strains of *Fusarium* spp., while trichothecenes were not found in cultures of two representative isolates of *F. equiseti* and one *F. sambucinum* isolate, reportedly the only two trichothecene-producing species among the ones analyzed [30]. Fumonisins were produced by strains of *F. proliferatum* and *F. nygamai*, in all of the three most important forms B1, B2, and B3. Particularly, the ITEM 19012 *F. nygamai* strainproduced significantly more FBs than the other strains. This particular strain also produced the highest levels of both BEA and MON. Indeed, members of *F. fujikuroi*

Table 4
Production of mycotoxins by representative isolates of major endophytic *Fusarium* species from potato root and tuber. None of the isolates tested produced either the type B trichothecenes deoxynivalenol, nivalenol or their respective acetylates or the type A trichothecenes diascetoxyscirpenol and T-2 toxin.

Species	Strain	Mycotoxin production (μg/g)[a]									
		Enniatins				BEA	Fumonisins			MON	FA
		B	B_1	A	A_1		B1	B2	B3		
F. nirenbergiae	ITEM 18972	n.d.	n.d.	n.d.	n.d.	396	n.d.	n.d.	n.d.	82	n.d.
	ITEM 18973	n.d.	n.d.	n.d.	n.d.	101	n.d.	n.d.	n.d.	63	n.d.
	ITEM 18983	n.d.	n.d.	n.d.	n.d.	1382	n.d.	n.d.	n.d.	66	n.d.
	ITEM 18981	n.d.	n.d.	n.d.	n.d.	952	n.d.	n.d.	n.d.	31	n.d.
	ITEM 18982	n.d.	n.d.	n.d.	n.d.	332	n.d.	n.d.	n.d.	27	n.d.
F. oxysporum	ITEM 18988	n.d.	n.d.	n.d.	n.d.	46	n.d.	n.d.	n.d.	21	n.d.
	ITEM 18941	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	105	n.d.
	ITEM 18946	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	ITEM 18987	n.d.	n.d.	n.d.	n.d.	525	n.d.	n.d.	n.d.	n.d.	n.d.
	ITEM 18942	n.d.	n.d.	n.d.	648	n.d.	n.d.	n.d.	n.d.	n.d.	156
	ITEM 18952	n.d.	n.d.	n.d.	446	7	n.d.	n.d.	n.d.	n.d.	n.d.
F. proliferatum	ITEM 18954	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	ITEM 18955	n.d.	n.d.	n.d.	n.d.	235	62	23	11	n.d.	n.d.
	ITEM 18994	n.d.	n.d.	n.d.	n.d.	58	3	2	2	n.d.	n.d.
F. nygamai	ITEM 18952	n.d.	n.d.	n.d.	n.d.	n.d.	5	2	2	n.d.	n.d.
	ITEM 18947	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	ITEM 18948	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	26	n.d.
	ITEM 18951	n.d.	n.d.	n.d.	n.d.	n.d.	105	29	24	67	n.d.
	ITEM 19012	n.d.	n.d.	n.d.	n.d.	4190	7946	4693	4333	538	n.d.
	ITEM 19013	n.d.	n.d.	n.d.	n.d.	41	49	18	13	4	n.d.
	ITEM 19014	n.d.	n.d.	n.d.	n.d.	n.d.	126	5	4	26	n.d.
	ITEM 18949	n.d.	n.d.	n.d.	n.d.	458	n.d.	n.d.	n.d.	75	n.d.
	ITEM 18950	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4	n.d.
F. equiseti	ITEM 18966	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	ITEM 18967	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F. sambucinum	ITEM 18958	n.d.	n.d.	n.d.	n.d.	21	n.d.	n.d.	n.d.	n.d.	n.d.

[a] amounts are the mean of two replicated cultures on sterile rice; BEA = beauvericin; MON = moniliformin; FA = fusaric acid; n.d. = not detected.

complex are known to produce multiple mycotoxins, including BEA, ENNs, FUMs, fusaproliferin, FA, fusarins, and MON [18]. Co-occurrence of MON with the cyclodepsipeptides BEA and ENNs has been also reported in *F. sporotrichioides*, *F. equiseti*, *F. avenaceum* and *F. oxysporum* [18].

Besides the regulated mycotoxins, we also investigated production of some "emerging" mycotoxins, which are neither routinely determined, nor legislatively regulated because of their lesser occurrence or uncertain toxicity in vivo. ENNs and BEA are toxic compounds similar for both structure and mode of action. They are cyclic hexadepsipeptides consisting of three D-α-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) alternating with three amino acid units and are biosynthesized by the non-ribosomal multifunctional enzymes enniatin and beauvericin synthetases. They both have ionophoric properties; they can form stable lipophilic complexes with cations and transport them into cell membrane and form cation-selective channels in membranes, thus impairing the membrane functions [91,92]. In potato, ENNs are virulence factors within the plant-pathogen interaction [93]. They have phytotoxic effects that include wilting and necrosis of tissues and are produced in vivo in potato tuber tissue infected by *Fusarium* spp [23]. However, their function as virulence factor seems to be host dependent [94]. Likewise, BEA showed strong phytotoxicity to tomato protoplasts, conceivably by inducing release of ascorbate from the cytosol to the apoplast of plant cell, with a consequent increase in cell wall plasticity that facilitates pathogen penetration [95]. BEA, along with FA, were contaminants of banana plants and fruits infected by *F. oxysporum* f. sp. *cubense* and proved to have phytotoxic effects on banana protoplasts [96]. In that study, virulence of *F. oxysporum* f. sp. *cubense* isolates correlated well with toxin accumulation. BEA was produced, in combination with unusual forms of ENNs, by fifteen *F. oxysporum* and two not-identified *Fusarium* sp. strains, out of twenty-eight *Fusarium* isolates from potato samples in Korea [97]. ENNs and BEA exhibit different toxic effects in vitro, including cytotoxicity and necrotic and pro-apoptotic effects to animal cell lines, but toxicity in vivo is generally low. This is thought to be due to their rapid metabolization rather than low bioavailability. MON is a phytotoxin, first isolated by Cole et al. [98]. Despite its name, nowadays MON is thought to be produced by a few *Fusarium* species, including *F. oxysporum*, *F. proliferatum*, *F. nygamai*, *F. equiseti* and *F. sambucinum*, but not by *F. moniliforme* [30]. MON is extremely soluble in water, due to its polarity, thus it is easily translocated through the plant. MON causes growth inhibition, necrosis, and chlorosis in many plants [99]. MON has a selective cytotoxicity in vitro, but shows severe effects in vivo, with symptoms of acute intoxication in test animals that include muscular weakness, respiratory stress, myocardial degeneration, and histopathological changes in kidneys, lungs, and pancreas and ultimately coma and death. FA has long been known as a wilt toxin [100] and is regarded as a virulence factor in plant tracheo-fusariosis caused by *F. oxysporum* ff. spp. in different crops [101–103], including potato [104]. FA is moderately toxic to animals, but it may potentiate the effect of other *Fusarium* toxins.

Although the above toxins are mainly regarded as phytotoxins that participate in the pathogenicity process, because of their occurrence in vivo and toxicity in animal models, they are emerging as possible concern for food safety. A recent study showed that BEA was the emergent mycotoxin with the highest prevalence in feed and feed ingredients, followed by ENNs [105]. Naturally, MON often co-occurs with ENNs and BEA, but also with trichothecenes, since several trichothecene-producing species produce MON, including *F. acuminatum*, *F. culmorum, F. equiseti*, and *F. sporotrichioides* [106]. FA is widespread on corn and corn-based food and feeds, frequently in association to other mycotoxigenic *Fusarium* species [107]. These toxins may contribute to the overall health risk of contaminated foods or feeds, either because of their direct toxic effects or because of additive or synergistic interaction with other co-occurring mycotoxins.

All the isolates we analyzed for mycotoxin production were endophytes of potato plants, recovered from healthy tubers or roots. Endophytic vs. pathogenic behavior of fungi is the outcome of a finely tuned balance, which depends on a molecular cross-talking between plant and symbiont [108]. Even if the molecular and physiological aspect of this interaction and its determinants are not completely clear, it is widely accepted that endophytism is a continuum which results from an equilibrium between fungal virulence and plant defense, and that endophytic behavior might revert to pathogenic, if one or more factors change [109]. In this sense, the chance that endophytic *Fusaria* become pathogenic and produce mycotoxins in tubers cannot be ruled out. Logrieco et al. [110] examined the production of zearalenone, zearalenols, trichothecenes and MON in cultures of some *Fusarium* species isolated from rotted potato tubers. They found that strains able to produce mycotoxins on autoclaved rice and maize kernels were not capable to produce toxins on fresh potato under any of the conditions tested. Conversely, Ellner [22] reported that potato tubers artificially infected with *F. sambucinum* were contaminated with the trichothecene toxin diacetoxyscirpenol in concentrations up to 200 μg/tuber, depending on the susceptibility of the cultivar tested. The toxin was also detected in tubers with no apparent disease symptoms. Likewise, Delgado et al. [21]studied the accumulation and diffusion of trichothecenes in potato tubers affected with dry rot caused by *F. graminearum* during storage. They found accumulation of either DON or NIV, depending on the *F. graminearum* genotype, in rotten tissue but not in the surrounding tissue. These findings suggest that contamination of potato tubers with mycotoxins in the field or at post-harvest, due to a change in lifestyle of endophytic microflora, is a potential risk that should be carefully considered and that is worth of further studies, along with possible means of control.

4. Conclusions

Plant-microbe endophytic association may bring a vast range of beneficial effects to the host plants. Endophytes produce biologically active metabolites with diverse activities, which interact with their host's physiology and ecology in different ways. These compounds can benefit host plants by protecting them against insect herbivores [111,112], by inhibiting plant pathogen growth and by eliciting host immune system against pathogens attacks [113]. On the other hand, some endophytes may actually become detrimental to their hosts, especially under stress conditions [109], or they may become pathogenic to crop plants other than their original host [114]. Thus, some endophytic fungi can be actually regarded as latent pathogens [8]. The chance that under certain conditions the endophytes of potato may change their lifestyle and become pathogenic to potato or other crop plants, should be considered.

Members of *Fusarium* are found worldwide, predominantly as soil inhabitants and plant pathogens, with the existence of nonpathogenic isolates [115,116]. In addition, some members of *Fusarium*, including some herein found to live endophytically in potato, are known to produce numerous toxins, some of which are regarded as virulence factors [93] or emerging mycotoxins (viz. ENNs, BEA, MON and FA) and others are regulated mycotoxins (viz. fumonisins), which may pose safety concerns for consumers. It is not yet well-established if, in certain conditions, the endophytic *Fusaria* may switch to a parasitic lifestyle and, in that case, if accumulation of toxins in the edible tubers can occur in the field or during storage. The findings of this and previous work [10], also open the prospect to conceivable strategies aimed at controlling pathogens of potato and, particularly, for biological control of mycotoxigenic *Fusaria*. Classical biological control is founded on the concept that most effective biocontrol agents should be sought amongst the coevolved antagonists of the target pathogen, which are supposed to have the highest specificity and biocontrol activity. In this regard, the report of the occurrence of fungi belonging to *Trichoderma* and *Clonostachys*, two well-known genera of biocontrol agents [117], as endophytes of potato in Iran [10] paves the way for studies on their use for sustainable control of potentially pathogenic endophytic fungi and for prevention of mycotoxin contamination of potatoes.

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Additional information

No additional information is available for this paper.

Data availability statement

Data will be made available on request.

The data and supportive information are available within the article. Data associated with the study have been deposited into publicly available repositories. Sequences were deposited in the GenBank database; strains were deposited in the microbial ITEM collection (http://server.ispa.cnr.it/ITEM/Collection/).

CRediT authorship contribution statement

Nasim Alijani Mamaghani: Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Data curation. **Mario Masiello:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Stefania Somma:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Antonio Moretti:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Hossein Saremi:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Miriam Haidukowski:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Claudio Altomare:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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