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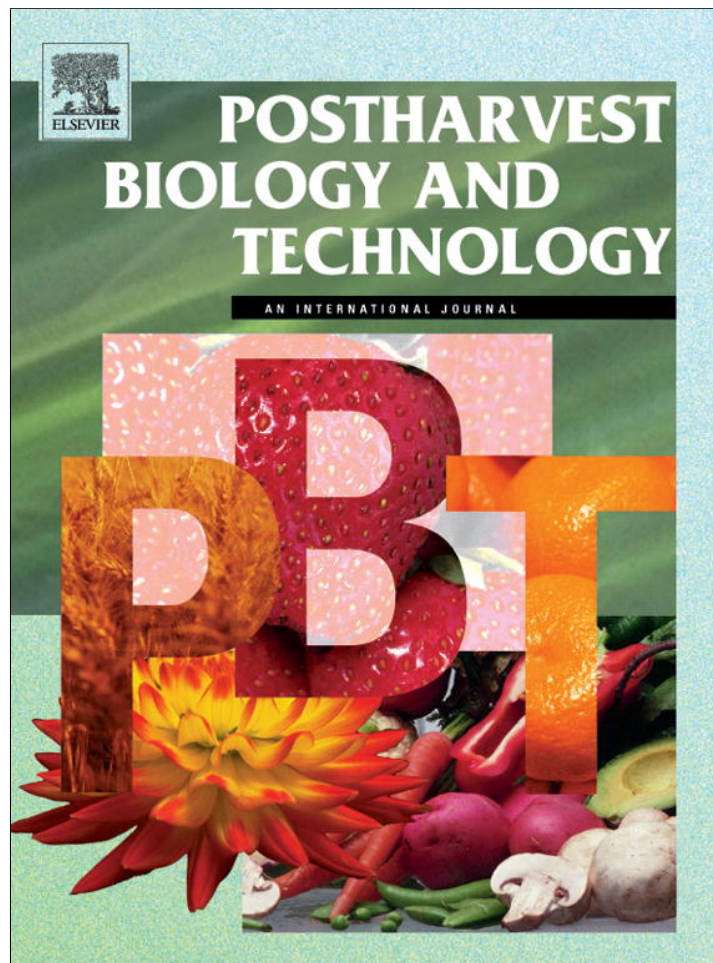
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Proteomic changes and endophytic micromycota during storage of organically and conventionally grown carrots

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ABSTRACT

The physiological state of carrot roots during extended cold-storage is decisive for high postharvest quality. We have investigated differences in the proteome and micromycota of organically and conventionally grown carrots during six months of storage. The levels of only 15 proteins changed in level during storage. Proteins involved in cold stress adaptation and cytoskeleton components changed; these changes in specific protein levels occurred mainly during the first month demonstrating adaptation to storage conditions and that the carrots were subsequently stable, indicating stable carrot quality. The changes observed were similar in the two cropping systems.

Using both biological isolation and a fungal PCR targeting the ITS region, we identified several endophytic species belonging to the Ascomycota. The most frequently encountered taxa were *Tetracladium*, *Leptodontidium*, *Nectriaceae* and *Phoma* which are known to occur as root endophytes or as root-associated fungi. As for the proteomics data, no consistent statistically significant differences in micromycota were observed between the two cropping systems. We conclude that cropping system did not have an influence on the postharvest quality of the carrots during six months of cold storage.

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1. Introduction

The year-round availability of quality carrots is important for the consumer market; therefore continuing technical improvements to extend cold storage are an important developmental challenge for the carrot industry. However, during cold storage, carrot roots become stressed due to the environmental conditions (low temperature and drought); these stress conditions induce modification of the metabolism of the roots and favour the development of certain diseases (Lecam et al., 1993; Kastelein et al., 2007; Louarn et al., 2012). At the same time, there is an increasing interest among consumers for food safety, especially with respect to pesticide residues and potential human pathogens. Furthermore, there is a general increased concern about the state of the environment, especially in relation to environmental impact caused by the excessive use of inorganic fertilisers. Consumers are therefore turning increasingly to organic products as their production does not entail the

use either of pesticides or mineral fertilisers (Woteki et al., 2001; Magnusson et al., 2003).

Relatively little is known about the physiological changes which occur during storage of root vegetables. Those demonstrated include changes in the occurrence of secondary metabolites, for instance, the isocoumarin, 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-MM) a phytoalexin (antimicrobial metabolite), which accumulates in carrots following pathogen infection and in response to treatment with various abiotic elicitors (Mercier et al., 1994; Jayaraj et al., 2009). Interestingly, the content of 6-MM from 1 month old stored carrots was significantly higher than after 4 and 6 months of storage (Louarn et al., 2012). Physiological changes due to cold stress (includes chilling (<20 °C) and freezing (<0 °C) temperatures) can also be observed at the protein level (Degand et al., 2009; Neilson et al., 2010). Several of these proteins e.g., dehydrin, heat-shock proteins (HSP) and anti-freeze proteins, are known to be involved in abiotic stress responses and have also been observed to accumulate following cold stress (Miernyk, 1997; Kawamura and Uemura, 2003; Griffith and Yaish, 2004; Renaut et al., 2004; Griffith et al., 2005).

Plant roots host a great variety of fungi; roots are not only susceptible to certain soil-borne pathogens, but can also be colonized by symbiotic mycorrhizae and endophytic micromycota.

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The spectrum of endophytic organisms present in a particular sample of roots will reflect the physiological state of the root and we assume that, if roots differ substantially in their physiological state, they will probably differ in their endophytic micromycota too. In carrot roots, several plant pathogen interactions have been studied (Kora et al., 2005; Kastelein et al., 2007; Louarn et al., 2012) as well as carrot mycorrhizal interactions (Dumas-Gaudot et al., 2004). Furthermore, Surette et al. (2003) investigated bacterial endophytes in carrots. However, no studies have been conducted on the endophytic micromycota of carrots roots. Endophytic fungi are asymptomatic for at least part of their life cycle but some can be latent pathogens or dormant saprophytes (that change lifestyle during host senescence, Promputtha et al., 2007; Yuan et al., 2010). The majority of endophytic populations are Ascomycetes (Gazizadeh and Chaverri, 2010; Botella and Diez, 2011) as well as the major carrot root pathogens (Farrar et al., 2004; Kastelein et al., 2007; Louarn et al., 2012). Potential benefits of host–endophyte associations include increased resistance to herbivores, pathogens and abiotic stress (Kuldau and Bacon, 2008; Porras-Alfaro and Bayman, 2011) and they can even, in some cases, be used as biological control agents. The root micromycota from plants grown in cold environments have been studied, thus the establishment of plant–fungal association in relatively extreme environments might help to understand the origins and the role of endophytes (Higgins et al., 2007; Rosa et al., 2010). Some endophytes, such as *Curvularia protuberata* in rice (Redman et al., 2011), are able to increase plant tolerance to cold stress. On the other hand, factors like soil pH, soil humidity, location and crop rotation (D'Amico et al., 2008), the use of fertilisers (Kernaghan et al., 2003) and predictably, the use of fungicides (Sieber and Grünig, 2006) can influence the diversity of the fungal endophytes communities, and especially nitrogen fertilisation might be one of the mechanisms by which agricultural management affects endophyte communities.

We have previously studied the response of organically and conventionally grown carrots at harvest and in response to pathogen attack during subsequent storage (Nawrocki et al., 2011; Louarn et al., 2012). Changes in specific defence-related metabolites including the accumulation of a phytoalexin (6MM) and proteins (PR-protein) were correlated to fungal infection. In contrast, the present study focuses on the differences between organic and conventional cropping systems and utilises physiologically independent assays to provide an objective evaluation as to whether there are indeed substantial differences in the way that organically and conventionally grown carrots develop during storage. Thus quantitative proteomics and sampling the endophytic Ascomycota of stored carrots provide two different assessments of the physiological state and, for the former, dynamics of the physiology of stored carrots. This study thus represents the first study of the endophytic Ascomycota in carrot roots. We show that, though there are some differences, the organically and conventionally cultivated carrots are substantially similar in quality, and that in essence the alterations in quality change concomitantly during storage.

2. Materials and methods

2.1. Plant material

The rotation experiment was started in the autumn of 2005 on a field grown organically since 1996, at Årslev, Denmark (10°27'E 55°18'N). The crops used in these rotations are representative of vegetables produced commercially in Denmark. The two cropping systems are described in detail in our previous study (Louarn et al., 2012; Thorup-Kristensen et al., 2012), and comprise a conventional system where synthetic fertilizers, pesticides, herbicides and fungicides were used according to normal practice and an organic

system. The roots were harvested in October 2007 and 2008, samples were taken on harvest day and the carrots then placed in cold storage at 1 °C and >98% RH and roots were sampled one, four and six months after harvest. Sampling was achieved by cutting two 15 mm cylinders per carrot with a cork borer; this cylinder was cut longitudinally in two equal pieces, one was kept for proteomic and micromycota analysis and the other for secondary metabolite analysis (Louarn et al., 2012). Only the upper 10 mm of this cylinder was used for analysis. Each sample consisted of 12 parts from six carrots giving approximately 12 g of fresh tissue per sample for each three biological replicates. The samples were frozen directly in liquid nitrogen and then freeze-dried.

2.2. 2D gel electrophoresis and image analysis

For the proteomic analysis, freeze-dried root samples were used from the 2008 harvest. Gel electrophoresis and image analysis were performed as described by Nawrocki et al. (2001, 2011) with some modifications according to Louarn et al. (2012). Where necessary, manual corrections of the protein spot detection and matching across the gels were made. Thus a 1.5-fold up or down regulation ($p < 0.05$) of the protein spot volume is taken as the cut-off for further analysis, since a lower level of change is inferred to approach the technical limitations of the technique. The significance of the differences between averaged spot volumes between pair-wise comparisons in organic samples compared to conventional samples at each time point was tested using Student's *t*-test, with $p < 0.05$. The same comparison and test was made between the samples at harvest time compared to samples stored 1 month, 4 months and 6 months for each cultivation systems. These two comparisons enable a comparison of the effect of cultivation system on the proteome changes and, independently, the effect of storage time on the proteome changes.

Protein spots whose intensity differed significantly were excised from the gels and digested with trypsin, and the proteins were identified using a 4800 Plus MALDI TOF-TOF Analyser (Applied Biosystems, MDS SCIEX) as in Louarn et al. (2012).

2.3. Micromycota

2.3.1. PCR identification

Freeze-dried samples of roots stored for one month, from the conventional and organic cropping system harvested in 2007 and 2008, were included. Roots cold-stored for one month were sampled in triplicate from the organic and conventional cropping systems both in 2007 and 2008. For DNA extraction, samples of freeze-dried carrot material were ground in liquid nitrogen and 20 mg was used for DNA isolated using the Nucleon Phytopure kit (Amersham Biosciences, Buckinghamshire, UK) following the manufacturer's instructions, except that 15 mg polyvinylpyrrolidone (Sigma) was added to the lysis buffer. The total reaction volume of the PCR reaction was 20 μ L and contained 1 \times Taq polymerase buffer including 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μ M each of the primers FITS-Fw1: GCCTGTC-GAGCGTCATT and FITS-R1: TCCTACCTGATYCGAGGTCAA (Fig. 1), 2 U Taq DNA polymerase (Amplicon, Copenhagen, DK) and 40–80 ng root DNA. The PCR was performed using a Mastercycler personal (Eppendorf). An initial denaturation at 95 °C for 3 min was followed by 36 cycles of denaturation at 94 °C for 20 s, annealing at 63 °C for 20 s, and extension for 20 s. The PCR was completed with a final extension step at 72 °C for 7 min. The PCR products were visualised on a 1.5% agarose gel. Following the manufacturer's instructions, the PCR products were cloned into the pGEM T-easy vector (Promega, Madison, USA) and transformed into DH5 α competent *Escherichia coli* cells (Invitrogen, Madison, USA). From each root sample in 2007 and 2008, respectively, 30 colonies and 25

Genus	Primer binding site (Fw)	Primer binding site (Rv)	
	gcctggttcgagcgtcatt	ttgacctcgratcaggtagga	
Ascomyc.	<i>Fusarium</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtagga JN672604
	<i>Glomerella</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtagga HE610587
	<i>Mycocentrospora</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg M.ac.2016
	<i>Phoma</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg GU004190
	<i>Pyrenochaeta</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg HM208713
	<i>Leptodontium</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg HQ623461
	<i>Sclerotinia</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg EU530000
	<i>Tetracladium</i>	gcctggttcgagcgtcatt	ttgacctcggatcaggtaggg FJ000373
Basidiomyc.	<i>Amanita</i>	gcctggttcgagcgtcatt	ttgacctcaaatcaggtagga JN020970
	<i>Agaricus</i>	gcctggttcgagcgtcatt	ctgacctcaaatcaggtagga FJ770403
	<i>Rhizoctonia</i>	gcctggttcgagcgtcatt	ctgacctcaaatcaggtagga HQ905443
	<i>Puccinia</i>	gcctggttcgagcgtcatt	aagacctcaaatcaggtagga GU598102
	<i>Ustilago</i>	gcctggttcgagcgtcatt	tcggacctcagattcaggtagga JN114418
	<i>Glomus</i>	gcctggttcgagcgtcatt	tcggacctcaaatcaggtagga JF439190

Fig. 1. Alignment of the FITS primers with sequences of Ascomycetes, Basidiomycetes and Glomeromycete (mismatches bases in white).

colonies were picked. Colony PCR with the vector primers T7 and Sp6 was performed as described by Sundelin et al. (2009). The PCR products were separated on an agarose gel and clones with band size of approximately 200 bp were sequenced using the sequencing service of StarSeq, Mainz, Germany. Sequences were queried against GenBank and identified in a BLAST search by the National Centre for Biotechnology Information (NCBI). Each sequence was identified to the lowest taxonomic rank common to the top blast hits. Only clone sequences represented at least twice in 2007 and/or 2008 were considered as identified taxa; those occurring only once were assigned to the phylum Ascomycota.

2.3.2. Fungi biological isolation

Fungi were isolated from stored organic and conventional roots. They were sampled from roots harvested and stored in 2009. Five roots from each sample were thoroughly rinsed under running tap water and two 15 mm cylinders centred through the root were removed from each root. The cylinders were surface disinfected 10 s in 70% ethanol, transferred to 2.5% NaOCl for 3 min and finally rinsed three times in sterile water. The disinfected cylinders were cut longitudinally and the outermost (1 mm) periderm layer cut off. The root pieces were placed on potato dextrose agar (PDA) and on malt extract agar (ME) both media were amended with Novobiocin (250 mg/L) and incubated at 16 °C and 20 °C. Fungi growing from the rotted tissue were purified on PDA. Fourteen fungi differing in colony morphology and spore morphology, seven of the isolates were non-sporulating, were selected for molecular identification based on ITS1-5.8s-ITS2 sequencing. For DNA isolation, the fungi were grown in potato dextrose broth at 100 rpm at room temperature for 14 days. The fungal biomass was harvested on nylon mesh (32 µm) and rinsed with sterilised water. The biomass was frozen at -80 °C and subsequently freeze-dried. DNA was extracted using Qiagen DNAeasy kit (Qiagen, Valencia, USA). The total reaction volume of the PCR reaction was 20 µL and contained 1 × Taq polymerase buffer including 1.5 mM MgCl₂, 0.3 mM of each dNTP, 0.5 µM each of the primers ITS1 and ITS4 (White et al., 1990), and 2.5 U Taq DNA polymerase (Amplicon, Copenhagen, DK). The PCR amplification was performed as described by Sundelin et al. (2009), except for the use of a Mastercycler personal (Eppendorf) PCR machine. For purifying PCR products: 5 µL PCR mixture was mixed with 10 U Exonuclease I, *E. coli* and 1 U FastAP Thermosensitive Alkaline Phosphatase (Fermentas, Helsingborg, Sweden) and incubated 15 min at 37 °C followed by 15 min incubation at 85 °C to stop the reaction. Sequencing and query search of the PCR products was performed as described above.

2.4. Statistical methods

The micromycota data were compared between the cropping systems, the comparison was made using the Mann–Whitney test for nonparametric testing. The statistics of the proteomic experiment (the Student's *t*-test) was performed using Image Master™ 2D Platinum software v.5.0 (GE Healthcare).

3. Results

3.1. Proteomics

The coverage of the proteome is approximately 1200 spots. The intensity of 10 spots increased and 5 decreased in response to cold storage (*p* < 0.05 and fold ≥ 1.5) in all the three replicates and both in the organic and conventional root (Table 1 and Fig. 2). No significant differences were observed between organic and conventional roots.

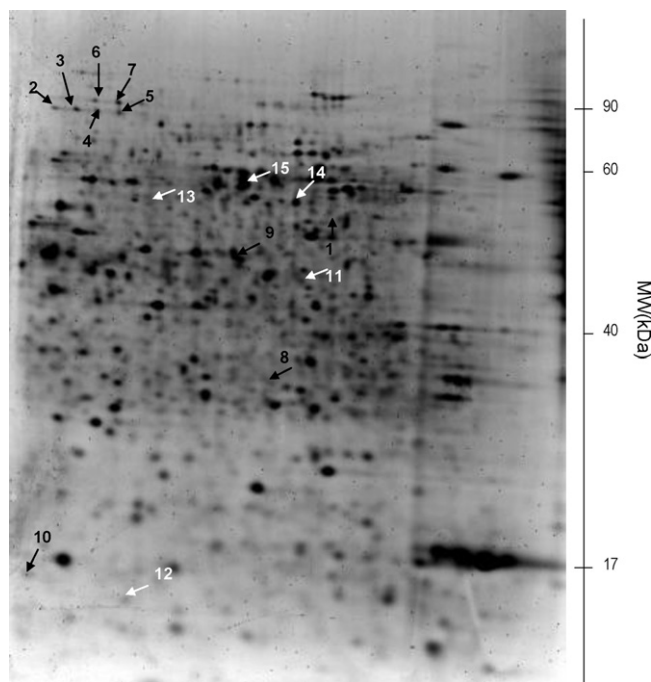


Fig. 2. Representative gel of carrot root (pl 4 to 7). The protein spots numbered in black are those that increased significantly during the storage and those in white decreased. The numbers in the gel images refer to protein numbers in Table 2.

Table 1
The effects of cold storage on the proteome of organically and conventionally cultivated carrots. Analysis of 2D-PAGE gels for proteins exhibiting more than 1.5 fold alteration in relative intensity following storage.

Spot number ^a	Fold changes in the conventional roots (in the organic roots)			Theoretical/observed MW (kDa)	Theoretical/observed pI	Accession number ^b	Protein name ^c	Organism	Biological process (molecular function) ^d	Matched peptides	Sequence coverage (%)	Score ^e	Sequence ^f
	Harvest/1 month	Harvest/4 months	Harvest/6 months										
1	1.9 (1.7)	2.3 (2.1)	2.3 (2.2)	51.187/49.902	5.52/5.28	GI:78099188	Probable L-ascorbate peroxidase 8	<i>Oryza sativa</i> subsp. <i>japonica</i>	Stress response (hydrogen peroxide removal)	7	26%	147	YPGITYADLFQLASATAIEE AGGPKIPMK 59 YGRVDVTAAEQCPPEGR 40 EVFYRMGLDDK (Mox) 65 MGLDDKEIVALSGAHTLGR (Mox) 71 FDNSYFKDIK 98 DQDLLVLPDAAALFEDPSFK 102 LSDLGAKFDPPEGFSLDDE PAVEEK 44 YLFAGVVDGR
2	1.6 (1.5)	1.8 (1.9)	2.1 (2)	84.589/83.978	6.51/6.77	GI:8134569	Methionine synthase	<i>Solenostemon scutellarioides</i>	Amino acid biosynthesis (methyl transfer)	4	6%	44	YLFAGVVDGR
3	2.1 (2.2)	2.5 (2.4)	2.5 (2.4)	84.589/83.981	6.51/6.659	GI:8134569	Methionine synthase	<i>S. scutellarioides</i>	Amino acid biosynthesis (methyl transfer)	5	6%	76	YLFAGVVDGR
4	1.7 (1.5)	2.1 (1.9)	2.4 (2.5)	84.821/84.459	6.27/6.58	GI:8134568	Methionine synthase	<i>Mesembryanthemum crystallinum</i>	Amino acid biosynthesis (methyl transfer)	4	7%	54	YLFAGVVDGR
5	1.5 (1.5)	1.7 (1.8)	1.7 (1.8)	84.821/84.528	6.27/6.41	GI:8134568	Methionine synthase	<i>M. crystallinum</i>	Amino acid biosynthesis (methyl transfer)	4	4%	68	YLFAGVVDGR
6	1.6 (1.7)	2 (1.9)	2 (1.9)	84.589/84.135	6.51/6.53	GI:8134569	Methionine synthase	<i>S. scutellarioides</i>	Amino acid biosynthesis (methyl transfer)	4	5%	66	YLFAGVVDGR
7	1.6 (1.7)	2 (1.9)	2 (1.9)	84.589/83.8857	6.51/6.42	GI:8134569	Methionine synthase	<i>S. scutellarioides</i>	Amino acid biosynthesis (methyl transfer)	4	6%	71	YLFAGVVDGR
												85	AGITVIQIDEAALR 55 SDEKLLSVFR 18 LLSVFR 71 YLFAGVVDGR
												85	AGITVIQIDEAALR 52 SDEKLLSVFR 68 YGAGIGPVVDIHSR

Table 1 (Continued)

Spot number ^a	Fold changes in the conventional roots (in the organic roots)			Theoretical/observed MW (kDa)	Theoretical/observed pI	Accession number ^b	Protein name ^c	Organism	Biological process (molecular function) ^d	Matched peptides	Sequence coverage (%)	Score ^e	Sequence ^f
	Harvest/1 month	Harvest/4 months	Harvest/6 months										
8	2.2 (2)	2.6 (2.5)	2.6 (2.5)	30261/29745	5.66/5.84	GI:3668173	Vegetative storage protein	<i>Arabidopsis thaliana</i>	Storage	2	13%	65	AYVEDYLITSK
9	1.7 (1.9)	2.2 (2.2)	2.2 (2.2)	47972/47854	5.57/5.29	GI:90110845	Enolase	<i>O. sativa</i>	Glycolysis	3	12%	104 74 81 95 102	NDTVNVWIFDLDDTLSSIPYYAK AVDNVNSVIAPALIGK LGNAILAVSLAICK QLVLPVPAPNVINGGSHAGNK SGSSSSSEDDGMGGR (Mox)
10	1.6 (1.8)	1.9 (2)	1.9 (2)	15356/15136	8.00/6.80	GI:57506540	Dehydrin	<i>Daucus carota</i>	Stress response	1	11%	102	
11	1.6 (1.5)	1.6 (1.5)	1.8 (1.7)	41735/41443	5.49/5.69	GI:1703108	Actin 7	<i>A. thaliana</i>	Cytoskeleton (structural molecule activity)	5	14%	59	VAPEEHPVLLTEAPLNPK
12	1.9 (1.6)	2.2 (2.1)	2.2 (2.1)	16547/15258	6.30/6.54	GI:1346675	Nucleoside diphosphate kinase 1	<i>A. thaliana</i>	Amino acid biosynthesis (role in synthesis of nucleoside triphosphates)	3	17%	22 16 109 76 119	LDLAGRDLTDSLMK LDLAGRDLTDSLMK (Mox) SSSSVKNYELPDGQVITIG AER NYELPDGQVITIG AER KIIGATNPAESAPGTIR
13	1.5 (1.6)	1.8 (1.8)	1.8 (1.8)	50.733/49.821	4.8/4.74	GI:267073	Beta-tubulin	<i>A. thaliana</i>	Cytoskeleton (structural molecule activity)	3	9%	130 64 114	IIGATNPAESAPGTIR GDFAIIDIGR PDNFVFGQSGAGNNWAK
14	1.8 (1.6)	2.3 (2.2)	2.3 (2.2)	49.823/49.752	5.19/5.62	GI:6094430	Alpha-tubulin	<i>Eleusine indica</i>	Cytoskeleton (structural molecule activity)	2	7%	49 82 56	FPGQLNSDLR NSSYFVEWIPNNVK LISQVSSLTSLR
15	2.5 (2.4)	2.7 (2.6)	2.7 (2.6)	53.029/50.854	6/5.78	GI:122239967	Ribulose biphosphate carboxylase	<i>D. carota</i>	Photosynthesis (photorespiration)	2	4%	70 53	FDGAINVDITEFQTNLVPYPR DTDILAAFR

^a Protein spot number as in Fig. 2.

^b Protein accession number derive from NCBI database.

^c Protein name derived from either Swissprot or NCBI databases.

^d Biological process the proteins is involved in and molecular function derived from either Swissprot or NCBI databases.

^e Ions mascot score for identified peptides by MSMS at $p \leq 0.05$.

^f Mox: oxidized methionine.

Table 2

List of the fungal taxa identified from ITS2 ribosomal DNA sequences of fungi cloned from samples of organic and conventional grown carrots in 2007 and 2008. Roots were cold stored at 1 °C for one month before sampling.

Taxa	Maximal identity (%)	2007		2008	
		Organic %	Conventional %	Organic %	Conventional %
<i>Leptodontidium orchidicola</i>	98–100	9	10	18	15
<i>Cadophora luteo-olivacea</i>	100	3	1	2	–
<i>Tetracladium</i> spp.	97–99	7	4	–	–
<i>Tetracladium maxilliforme</i>	98–100	24	19	13	17
<i>Tetracladium furcatum</i>	97–100	10	10	11	4
<i>Peyronellaea</i> spp./ <i>Phoma</i> spp. ^a	100	0	8	2	–
<i>Phoma eupyrena</i> /P. <i>herbarum</i>	99–100	6	5	10	2
<i>Epicoccum sorghinum</i> /Phoma spp.	100	3	3	–	–
<i>Davidiella</i> ssp./ <i>Cladosporium</i> ssp.	100	–	1	3	6
<i>Ilyonectria macrodidyma</i>	99–100	3	–	5	8
<i>Ilyonectria macrodidyma</i> /I. <i>radicicola</i>	98–99	–	–	2	2
<i>Ilyonectria radicola</i> /Ilyonectria spp. ^b	100	–	1	7	6
<i>Giberella avenacea</i> /Fusarium <i>tricinctum</i>	99	3	4	2	8
<i>Gibberella moniliformis</i> /G. <i>thapsina</i>	100	3	–	–	–
<i>Bipolaris</i> sp./ <i>Curvularia clavata</i>	98–100	10	7	–	–
<i>Pyrenochaeta</i> sp.	100	1	1	2	4
<i>Dendryphon nanum</i>	100	–	–	3	2
<i>Geomyces</i> sp.	96	–	–	–	6
<i>Geomyces pannorum</i>	100	–	1	2	4
<i>Geomyces leuteus</i>	100	–	–	–	4
<i>Glomerella acutata</i> ^c	99–100	–	7	2	2
<i>Rhexocercosporidium</i> sp.	98	–	–	3	–
<i>Rhexocercosporidium carotae</i>	99–100	4	7	–	6
<i>Marssonina brunnea</i>	83	3	–	–	–
<i>Apiognomonina errabunda</i>	100	–	1	2	2
<i>Dothideomycetes</i>	93	–	3	–	–
Ascomycota ^d	86–100	11	5	11	6
Number of isolates		n=70	n=73	n=61	n=53

^a Significant difference ($p < 0.05$) in recovery of *Peyronellaea* spp./*Phoma* spp. between the cropping systems in 2007 using the Mann–Witney test. No other comparisons differed significantly.

^b Best hits included either *I. rufa*, *I. crassa* or *I. robusta* all having the same maximal identity as the dominating hits of *Ilyonectria radicola*.

^c For 3 isolates the blast search could not distinguish *G. acutata* from *G. fioriniae*.

^d Sequences of ascomycetes only occurring once in 2007 and 2008 were identified to the following taxa: *Aureobasidium pullulans*, *Fusarium equiseti*, *Paraphoma chrysanthemicola*, *Aspergillus* spp., *Zalerion varium*, *Cochliobolus lunatus*, *Leptosphaeria* sp., *Cadophora* sp., *Glarea* sp., *Penicillium* spp., *Spirosphaera cupreorufescens*, *Epicoccum nigrum*, *Cladosporium sphaerospermum*/C. *cladosporioides*, *Fusarium merismoides*, *Exophiala* sp., *Heliotiales*.

Only two of the 15 proteins identified matched directly to carrot protein sequences in the databases. Most of the proteins identified were at their predicted position in the gels (based on MW and pI) but some of the identified proteins are inferred to be degraded since their positions in the gels did not correspond to the theoretical pI or MW (Table 1 and Fig. 2). All spot identifications in Table 1 are based on two or more peptides except for spot 10 which is based on a single peptide: it is conceivable that this therefore represents contamination of the major spot from a protein in the background which is unaffected by cold storage. Among the 15 identified proteins, three of them are part of the cytoskeleton (spots 11, 13 and 14), and three others are involved in stress responses (spot 1, 10 and 12).

3.2. Micromycota

In the roots harvested in 2007, 143 different fungal isolates were identified as belonging to 22 taxa and 114 isolates identified to 21 taxa in the roots from 2008 (Table 2). The occurrence of each fungal taxon in the organic and conventional root were compared. In 2007, only *Peyronellaea* spp./*Phoma* spp. differed significantly in occurrence; this taxon occurred more often in the conventional roots than in the organic ones. No significant difference could be attributed to the cropping systems in 2008. Representatives from the nine most frequently occurring taxa were identified in both years (Fig. 2) and from most samples. This reproducibility supports the conclusion that these data are robust (see supplementary Tables 1 and 2). However, frequencies of occurrence in our samples varied considerably, e.g., 7% *Geomyces* was found in 2008 against 1% in 2007 and the same was seen for *Davidiellaceae*. On

the other hand, many of the other groups like *Tetracladium*, DSE, Pleosporaceae, Nectriaceae and Didymellaceae were consistently represented with high frequencies in both years. Averaged over year and cropping system, *Tetracladium*, *Leptodontidium*, Nectriaceae and Didymellaceae were the most frequently isolated taxa, respectively, representing 31%, 14%, 13% and 10% of all isolates.

Culturable fungi were isolated from stored roots in 2009. Based on ITS 1–2 sequences, the following taxa were identified *Tetracladium furcatum*, *Tetracladium maxilliforme*, *Leptodontidium* sp., *Ilyonectria* spp., *Fusarium solani*, *Plectosphaerella cucumerina* (syn. *Fusarium tabacinum*), *Phoma* spp., *Pyrenochaeta* sp., *Acremonium strictum*. All the fungi identified from biological isolation were Ascomycetes.

4. Discussion

We have demonstrated in this study, using two completely independent methods to monitor physiological quality, that no substantial differences have arisen between organic and conventionally cultivated carrots during storage.

At the protein level, three proteins that changed significantly during storage are involved in stress responses. The increase of ascorbate peroxidase (spot 1) correlated with its involvement in response to abiotic and biotic stress; this protein has been reported previously to accumulate following oxidative stress in roots due to cold treatment (Neilson et al., 2010). Interestingly, enhanced peroxidase activity might have an influence on bacterial as well as fungal endophyte populations. A dehydrin (spot 10) also accumulated and has been reported previously to be induced by drought,

salinity or/and low temperature (Close, 1996) and might stabilize cell membranes and protect other proteins from denaturation during dehydration (Allagulova et al., 2003). Dehydrin accumulation has been shown in proteomic studies of cold stress in *Arabidopsis* (Kawamura and Uemura, 2003; Renaut et al., 2004). It might also play a role in conjunction with molecular chaperones, DNA binding and repair, or participate in the cytoskeleton (Rahman et al., 2011). Tubulins (alpha and beta) are the major constituents of microtubules. Both alpha- and beta- tubulin levels (spots 13 and 14) decreased during cold storage. The reduced abundance of tubulins correlates with the appearance of cold-stable microtubules which is linked with low temperature stress (Abdrakhamanova et al., 2003). Actin 7 (spot11), another cytoskeleton component decreased during storage. The cytoskeletal proteins assemble into polymers that play an important role in the cell structure and are determinant of the cell shape; during cold adaptation, a reorganisation of these polymers is necessary to preserve the cell integrity. The presence of RuBisCo (spot15) in roots is surprising, because this is a non-photosynthetic organ but it has previously been reported in roots (Suzuki et al., 2009; Louarn et al., 2012). A CO₂-binding role for RuBisCo has been demonstrated in developing *Brassica napus* embryos (Schwender et al., 2004). RuBisCo might have a similar function in roots with respect to the expression of decarboxylating enzymes of the Krebs cycle (Mooney et al., 2006). A decrease of RuBisCo could indicate a slowing down of the energy metabolism. Vegetative storage protein (spot 8) levels increased and suggests an adaptation of the roots in order to store energy for the next year; carrot is a biannual crop plant and can thus withstand a period of cold/freezing temperatures over winter.

The fungal communities of cold-stored carrot roots were identified by rDNA clone libraries that enabled us to identify 257 Ascomycetes isolates (clones) in the roots. The FITS PCR-primers used exhibit greatest affinity for detection of Ascomycetes and would not be expected to yield Basidiomycetes or Glomeromycetes due to mismatches at the primer binding sites (Fig. 1). Although the FITS reverse primer has a single mismatch at the 5' end for several Ascomycete genera, it does not seem to affect the efficiency of the primers to amplify, e.g., *Tetracladium* and *Leptodontidium*. The robustness of the DNA based method was confirmed by the result of the biological isolation that yielded solely Ascomycete taxa, all bar one was also detected by the FITS primers. In general, ascomycetes also seem to constitute the majority of endophytic populations in roots of other species studied (Upson et al., 2009; Andrade-Linares et al., 2011). Concerning the absence of Glomeromycetes, these are known to be associated with carrots but most frequently with the fine roots which were not sampled in this study (Dumas-Gaudot et al., 2004). There was no significant effect of the cropping system on the micromycota community, only *Peyronellaea* spp./*Phoma* spp. significantly differ (Table 2). In contrast Wilberforce et al. (2003) found that agricultural management practice affected communities of root-endophytic fungi in grasslands at different locations (Wilberforce et al., 2003). However, in our field experiment, the plots were situated at the same location.

The nine most frequently occurring taxa were identified in both years but the isolation frequencies of clones varied considerably between 2007 and 2008 (Fig. 2). These differences could be related to the relatively small sample size (257 clones), the varying climatic conditions or the use of different seed lots in the two years. The occurrence of several taxa only once, either in 2007 or in 2008, can be attributed to the same factors (Table 2). These pathogens can survive inside carrot roots (or carrot debris) in the field and might become inoculum sources that will infect a later crop. The taxon *Leptodontidium orchidicola* is frequent in both years and *Cadophora* spp., belong to the artificial group "dark septate endophytes" (DSE) which occur widely in association with plant roots in cold stressed habitats (Upson et al., 2009; Rosa et al., 2010) (Table 2). The DSE

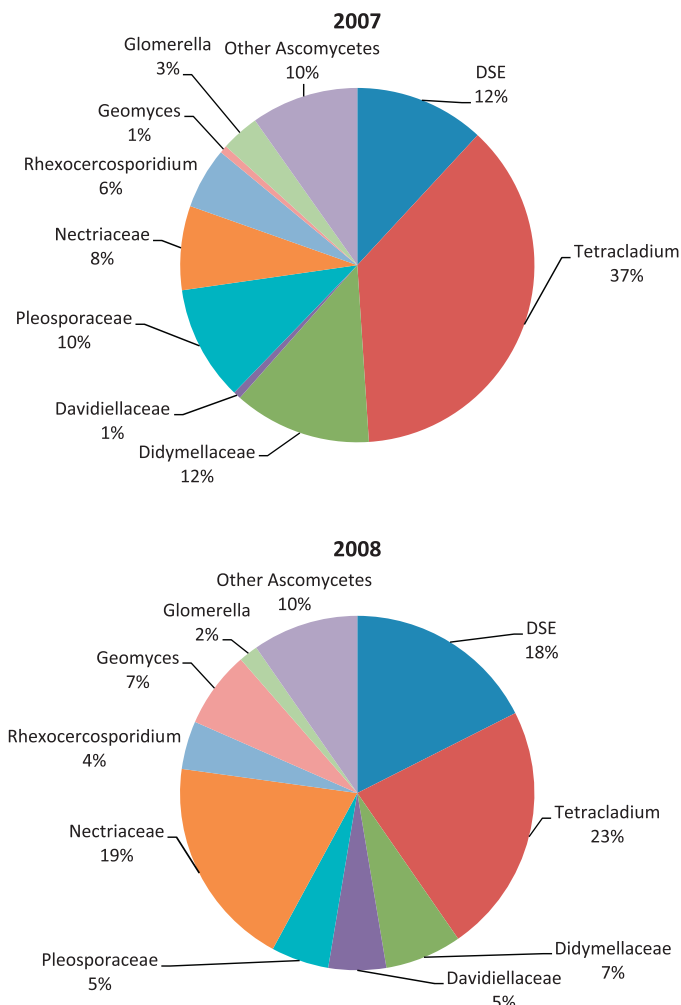


Fig. 3. Proportion of fungal taxa recovered from DNA sequence analysis of cold stored carrots in 2007 and in 2008. DSE (dark septate fungal endophytes) contains *Leptodontidium* and *Cadophora*. Nectriaceae contains *Fusarium*, *Giberella* and *Ilyonectria*. Pleosporaceae contains *Bipolaris*, *Pyrenochaeta* and *Dendryphion*. Davidiellaceae contains *Davidiella* and *Cladosporium* and, Didymellaceae contains *Phoma* and *Peyronella*.

have received much attention since they can stimulate host growth and protect plants against various stress conditions, e.g., by secretion of secondary metabolites (Schulz et al., 2002; Porrás-Alfaro and Bayman, 2011). Other taxa *Geomyces* spp., and *Davidiella* spp. detected in the cold-stored carrots have also been identified as endophytes in roots of plant species adapted to cold conditions (Rosa et al., 2010) (Fig. 3).

Some of the identified taxa, e.g., *Rhexocerosporidium carotae* (Kastelein et al., 2007) and *Fusarium* spp., are known as carrot postharvest pathogens. However, they remained latent as no visible symptoms developed during the 6 months of cold storage (Table 2). This could be due to defence mechanisms like the accumulation of the antioxidative ascorbate peroxidase which can protect against necrotrophs (Table 1) induced either by the cold environment or by endophytic species.

The changes observed at the protein level indicate an adaptation of the roots to the cold storage conditions within the first month of storage after which the root proteome was stable for the remaining 5 months. Both organic and conventional roots were still marketable after 6 months of cold storage. The observed changes of the proteome could be associated with a cold adaptation of the carrots. We presumably did not isolate all the fungal endophytes taxa but with over 257 clones identified, representing

approximately 40 taxa from a total of 72 carrots, we believe these samples are representative of the micromycota. The fact that some pathogens were identified as symptomless endophytes might be due to activation of defence mechanisms induced either by the cold environment or certain endophytes. We conclude that cropping system did not have an influence on the postharvest quality of the carrots during six months of cold storage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.postharvbio.2012.08.011>.

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