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Short Note

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Endophytic yeasts and filamentous fungi associated with southern Brazilian apple *(Malus domestica)* orchards subjected to conventional, integrated or organic cultivation

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Endophytic fungi were isolated from leaves, flowers and fruit of healthy apple trees (*Malus domestica*, BORKH.) growing in southern Brazilian orchards under three different cultivation systems (conventional, integrated and organic), during two vegetative cycles. The greatest total number of endophytic isolates was obtained from the orchards under organic cultivation when compared to integrated and conventional cultivation systems. Filamentous fungi from the genera *Colletotrichum*, *Xylaria* and *Botryosphaeria* were the most frequent ones and the most representative yeast genera were *Sporobolomyces*, *Rhodotorula*, *Debaryomyces* and *Cryptococcus*. It is suggested that some isolates may be used as indicators of the different management systems.

Maintenance of the microbiota which inhabit the phylloplane and the internal organs of plants is of extreme importance for the continuity of the ecological interactions between the organisms and equilibrium of agroecosystems. Agricultural activity resulting in manipulation of agroecosystems tends to cause a reduction in the size and diversity of microbial populations. The identification and monitoring of agroecosystem microbiota can help in the development of agricultural production and management systems which lead to the implementation of practical measures which can minimize the disequilibrium between humankind and nature, such practices being an extremely important factor in determining the quality of agroecosystems (BODDY and WIMPENNY 1992).

The majority of apple producers in Brazil, use conventional cultivation systems based on chemical pesticides and inorganic fertilizers. Since 1996, some producers opted to the integrated cultivation system. A third system designated organic system was implanted in 1994. The result of producing apples using integrated and organic cultivation systems is that the resultant fruits have lower levels of chemical residues, but little scientific data is available on the quality of these agroecosystems. One way to estimate the impact production systems have on the environment is to analyze the internal biological diversity of plants growing under the different systems, as represented by the endophytic fungi population (ISAAC 1992, REDLIN and CARRIS 1996). Sustainability of the three apple productions systems have been

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compared (REGANOLD *et al*. 2001) with no observable differences in pests, diseases or physiological disorders among plots. The present study aimed to quantitatively and qualitatively assess the population of both endophytic yeasts and filamentous fungi associated with apple trees under conventional, integrated and organic production systems.

Biological material and samples: The leaves, flowers and fruit of apple trees were collected from orchards in the brazilian state of Rio Grande do Sul, this region having a subtropical humid climate with an average temperature of 22.6 °C in the hottest month (January). Collections were done in two conventionally-managed orchards (1 and 2 at 28°29′11″ S, 51°20′09″ W), two integrated-managed orchards (3 and 4 at 28°25′17″ S, 50°53′22″ W) and two organically managed orchards (5 at 28°52′08″ S, 50°26′06″ W and 6 at 28°45′35″ S, 51°20′09″ W). Samples were collected during October to March for two vegetative cycles (cycle 1 : 2000/2001 and 2 : 2001/2002). Flowers were collected in October, fruits monthly from November to February and leaves monthly from October to March, from 30 randomly-chosen trees being 5 from each one of the 6 orchards during the first cycle and from 42 trees, during the second cycle. From each tree, 10 flowers were collected and from them 48 flower fragments used, being 3456 fragments evaluated. Two fruits collected from each plant were used per sample, 16 fragments analyzed from each fruit. a total of 9216 fragments being evaluated. From each plant 8 leaves were collected, two fragments of each used, and a total of 13824 fragments analyzed.

Isolation and identification of endophytic yeasts and filamentous fungi: Endophytes were isolated according by a modification of RODRIGUES and SAMUELS (1999). Leaves and fruit were washed in distilled water and surface disinfected by immersion in 96% (v/v) ethanol for one minute and then in sodium hypochloride (3% available chloride) for 4 minutes and rinsed three times for 1 minute in sterilized distilled water. After disinfection the principal nerve of the leaves was cut into 5–8 mm fragments and 0.5 cm^2 samples removed from the external, central and internal pericarp of the fruits. Flower-petals were washed and surface disinfected as above except that 50% (v/v) ethanol and 0.06% (w/v) sodium hypochloride (1.5% available chloride) were used. Endophytes were isolated by plating groups of onto SABOURAUD Dextrose Agar (SDA) in plates supplemented with 100 µg/ml tetracycline. Half of the plates were incubated at 28 °C and the other at 37 °C, both under a 12-hour light/dark photoperiod, for 20 days and the number of yeast colonies and filamentous fungi were counted and one morphotype of each was isolated. Fungi were purified and transferred to Potato Dextrose Agar (PDA) (MERCK) slants. Filamentous fungi isolates were identified according to standard taxonomic keys. Yeast were identified according to the methods described by KURTZMAN and FELL (1998) and BARNETT *et al*. (1990). After identification the yeasts were grouped into classes (PENNYCOOK and NEWHOOK 1981) as non-pigmented and pigmented.

Colonization frequencies and statistical analysis: The colonization frequency (CF%) of an endophyte was calculated according to the method of HATA and FUTAI (1995) where: CF% = Nc/Nt being Nc = number of fragments colonized by each endophyte and *Nt* = total number of fragments observed; Analysis of variance (ANOVA) was used to analyze the data and the means compared by the Tukey-test using version 10.0 of the SPSS program.

Total number of obtained endophytes is shown in Table 1. More isolates were recovered at 28 than at 37 °C, agreeing with previous reports (FISHER *et al*. 1992).

For both endophytic yeasts and filamentous fungi the number obtained from organically managed apple trees was higher than those obtained from conventional or integrated systems, irrespective of the type of tissue (leaf, flower or fruit) sampled (Table 2).

The population of endophytic yeasts and filamentous fungi obtained from leaf (Fig. 1) and fruit fragments (data not presented), show that the later the samples were collected, the greater was the endophytic population, coinciding with leaf senescence and fruit maturation as previously described (JOHNSTON 1994, RODRIGUES 1994, RAJAGOPAL and SURYANARAYANAN 2000, KUMARESAN and SURYANARAYANAN 2002).

The fungi isolated and identified were allocated to nine groups, three (*Alternaria, Botryosphaeria* and *Cladosporium*) being dematiaceous and six (*Colletotrichum* species 1 and 2, *Epicoccum*, *Fusarium*, *Xylaria* and *Mycelia sterilia*) non-dematiaceous, the most fre-

Table 1
Total number of endophytic yeasts and filamentous fungi obtained at 28 and 37 °C from pooled samples (leaf, flower and fruit fragments) taken from apple trees during two vegetative cycles, grown under three different production systems

Isolation temperature (°C)	Number of endophytic isolates					
	Production system					
	Conventional		Integrated		Organic	
	filamentous fungi	yeast	filamentous fungi	yeast	filamentous fungi	yeast
28 °C	394	38	314	38	1085	108
37 °C	60	22	51	12	124	68
Total	514		415		1385	

Table 2
Frequencies (CF) of flower, leaf and fruit fragments producing yeasts and filamentous fungi obtained from conventional, integrated and organic production systems apple trees, during two vegetative cycles

Production system	Filamentous fungi			Yeasts		
	Leaf	Flower	Fruit	Leaf	Flower	Fruit
Conventional	0.3^{a}	0.08^{a}	0.01^{a}	0.03^{a}	0.04^{a}	0.01^{a}
Integrated	0.3^{a}	0.09^{a}	0.01^{a}	0.04^{a}	0.03^{a}	0.01^{a}
Organic	0.8^{b}	0.12^{b}	0.04^{b}	0.09^{b}	0.10^{b}	0.05^{b}

Means with the same superscript letter in the same column do not differ significantly by the Tukey-test ($P \leq 0.05$)

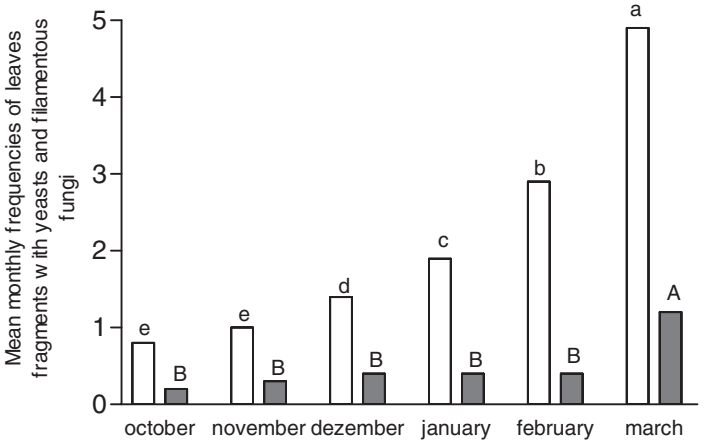

Fig. 1
Distribution of endophytic yeasts and filamentous fungi obtained from the leaves of apple trees. Means with different capital letters (yeasts) and different small letters (filamentous fungi) are significantly different by the Tukey-test ($P < 0.05$)
■ yeasts □ filamerntous fungi

Table 3
Colonization frequency (CF%) of isolation of different genera of endophytic filamentous fungi from fragments of leaves and fruit (pooled data) of apple trees cultivated in different production systems collected during two vegetative cycles

Genus	Type of Fragment							
	Leaf				Fruit			
	Production system				Production system			
	Conventional	Integrated	Organic	Total	Conventional	Integrated	Organic	Total
Alternaria	0.8	0.3	1.4	2.5	–	–	–	–
Botryosphaeria	1.8	2.0	3.4	7.2	0.04	0.04	0.4	0.48
Cladosporium	1.0	0.4	2.4	3.8	–	–	–	–
Colletotrichum sp. 1	1.7	2.0	9.5	13.2	0.08	0.08	0.5	0.66
Colletotrichum sp. 2	1.5	1.0	1.1	3.6	0.08	0.04	0.08	0.2
Epicoccum	0.4	0.4	2.0	2.8	–	–	0.13	0.13
Fusarium	0.7	0.6	0.8	2.1	–	–	–	–
Xylaria	1.0	1.7	9.8	12.5	0.04	0.04	0.9	0.98
Mycelia sterilia	0.2	0.3	1.4	1.9	–	–	0.04	0.04

Table 4
Pigmented and non-pigmented yeasts isolated from leaf, flower and fruit fragments obtained from apple trees cultivated using three different types of production system during two vegetative cycles

Yeast	Production system								
	Conventional			Integrated			Organic		
	Leaf	Flower	Fruit	Leaf	Flower	Fruit	Leaf	Flower	Fruit
Pigmented									
Sporobolomyces roseus (KLUYVER & VAN NIEL)							+*		
Sporodiobolus pararoseus (FELL & TALLMAN)	+			+			+		
Rhodotorula mucilaginosa (JÖRGENSEN) HARRISON	+			+			+		
Non-pigmented									
Debaryomyces hansenii (ZOPF) LODDER & KREGER-VAN-RIJ	+	+		+			+	+	
Candida spp. BERKHOUT						+	+		+
Cryptococcus laurentii (KUFFERATH) SKINNER	+			+	+		+	+	
Cryptococcus spp. VUILLEMIN									+
Pichia spp. E. C. HANSEN						+			

* + : present

quently ones belonging to the genera *Colletotrichum*, *Xylaria* and *Botryosphaeria* (Table 3). *Colletotrichum*, *Xylaria*, *Fusarium* and *Mycelia sterilia* are fungi usually found in association with plants including the tropical ones (PEREIRA *et al*. 1993, 1999, RAJAGOPAL and SURYANARAYANAN 2000, GAMBOA and BAYMAN 2001, PHOTITA *et al*. 2001).

Yeast genera isolated (Table 4) were obtained mainly from leaf fragments, independent of the production system used. There appeared to be no great difference in between the different cultivation systems in respect of the diversity of the genera recovered, except that *Sporobolomyces roseus* was isolated only from leaves taken from organically cultivated plants, and the genus *Pichia* only from fruit cultivated according to an integrated management system.

In the light of these results it appears that chemical compounds, mainly fungicides which are used in the conventional and integrated production systems reduce the endophytic fungi populations of apple trees. Although results were derived only from two vegetative cycles, it may be suggested that some endophytic microorganisms could be used to detect and to confirm the status of different production systems orchards. For instance, *Sporobolomyces roseus* isolated only from leaves of organic orchards and the genus *Pichia* isolated only from fruits collected in integrated production systems may be used as indicators of these management systems.

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