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Effects of a biocontrol agent of apple powdery mildew (*Podosphaera leucotricha*) on the host plant and on non-target organisms: an insect pest (*Cydia pomonella*) and a pathogen (*Venturia inaequalis*)

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A number of studies have focused on the selection and use of new biocontrol agents, but the effects of the introduction of these microorganisms on non-target organisms, including the crop plants themselves, are not well known. Non-target effects of sprayed applications of a potential biocontrol agent of apple powdery mildew (*Podosphaera leucotricha* Ell. Et Ev.), on scab infections (*Venturia inaequalis* Cooke Winter), on codling moth [*Cydia pomonella* L. (Lepidoptera: Tortricidae)] oviposition and damage and apple (*Malus x domestica*) fruit quality are examined. This biocontrol agent, an epiphytic yeast isolate called Y16, affected neither conidia germination of *V. inaequalis* nor their penetration of the leaf tissue but suppressed the disease caused by this pathogen. The quantity of eggs laid by the codling moth during its second flight period on yeast treated trees was significantly different to the quantity of eggs laid on the untreated trees. In the first season of the experiments, more eggs were laid on the treated trees, especially on those tree parts closest to the fruit. These results, however, were not confirmed the following season: fewer eggs were laid on the treated trees than on the untreated trees. These conflicting observations are attributed to year-to-year variation in environmental conditions, which may affect yeast survival and activity. A 2-month-long assay was conducted in the orchard during the codling moth's second flight period from mid-July until mid-September. The yeast treatment did not affect the damage caused by the codling moth to the fruits. Finally, the yeast treatment did not affect any of the examined fruit quality parameters.

Keywords: conidia germination; apple scab; codling moth; oviposition; fruit quality

Introduction

Currently, apple production systems require a large number of chemical applications in Europe, from about 10 up to 25 each year. The most important insect pest of this

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crop is the codling moth (*Cydia pomonella* L.); and the most important diseases of this crop are apple scab (*Venturia inaequalis* Cooke Winter) and the powdery mildew (*Podosphaera leucotricha* Ell. Et Ev.), against which the majority of chemical treatments are addressed. Over the last 10 years, codling moth and *V. inaequalis* have begun to develop resistance to several chemical treatments (Mattedi and Varner 2000).

In recent years, the development of new biocontrol agents has intensified in response to the development of resistance against chemical pesticides, and environmental and consumer concerns which discourage the use of chemicals. Very few products based on biocontrol agents are commercially available to control pathogens such as powdery mildews (Elad and Freeman 2002). Epiphytic yeasts, saprophytic bacteria and filamentous fungi are common inhabitants of the plant surface (Elad, Köhl and Fokkema 1994), but the introduction of new epiphytic microorganisms onto leaf and fruit surfaces may have unforeseen positive or negative consequences. For example, such introductions could induce plant resistance mechanisms (Blakeman and Fokkema 1982; De Meyer, Bigirimana, Elad and Höfte 1998), or trigger physiological changes in plant organs (Elad 1996), affect the fruit yield and quality (Swadling and Jeffries 1996), induce susceptibility against non-target diseases, affect the phyllosphere microflora community qualitatively and quantitatively (Moris and Rouse 1985; Elad, Baker, Faull and Taylor 2004), modify the plant volatile emission (Darriet et al. 2002) and/or change the leaf surface chemical composition (Andrews 1992). Modes of action of biocontrol agents also include competition for nutrients needed for the pathogen development (Mercier and Lindow 2000) and production of antimicrobial compounds (McCormack, Wildman and Jeffries 1993), which may include the emission of substances that affect the development of the pathogen and/or directly interfere with the multiplication of the pathogen (Hatcher 1995; Guetsky, Shtienberg, Elad, Fischer and Dinoor 2002). These reactions and modes of action may, in turn, affect the development of pests and pathogens whose biology or behaviour is dependent on specific aspects of the plant's physiology.

Several biocontrol candidates against the biotrophic phase of *V. inaequalis* have been identified. Isolates of *Cladosporium* or *Fusarium* (Fiss et al. 2000), as well as isolates of *Acremonium* (Gessler, Reidy, Lotscher and Schloffer 2000), *Athelia* and *Chaetomium* (Heye and Andrews 1983) have been reported to be capable of inhibiting apple scab. Similar biocontrol candidates have been shown to have an impact on arthropod pests. For instance Martin, Bedel de Buzareingues, Barry and Derridj (1993) showed a relationship between the introduction of a yeast to the leaf surfaces of maize and a drop in the quantity of eggs laid by the European corn borer *Ostrinia nubilalis* Hb. (*Lep., Pyralidae*) on these treated surfaces. Very little is known about the effects of biocontrol agents on non-target insects. In general, the effects of these introduced microorganisms on non-target organisms, including the crop plants, are poorly understood.

An epiphytic yeast, isolate Y16, is currently under study as a biocontrol agent of powdery mildews (Y. Elad and I. Pertot, personal communications). This micro-organism was isolated in Israel from lavender plants (*Helichrysum gymnocephalum*) by the second author. The yeast strain 16 was selected for its ability to survive in the apple tree canopy and to suppress different powdery mildews (Mendelsohn, Rav David, Elad and Shtienberg 2007; Rav David et al. 2007), including the grape

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powdery mildew caused by *Erysiphe necator* Schwein (Mendelsohn, Elad, Rav David and Ovadia. 2006) and apple powdery mildew (causal agent *P. leucotricha)* (Alaphilippe, Elad, Derridj and Gessler 2007). Preliminary experiments show induced resistance as possible mode of action (Y. Elad, personal communication). In this paper, we examine the effects of the introduction of Y16 to apple tree, on the non-target organisms apple scab and the codling moth, and the host plant fruit production.

Concerning the effect of the yeast on apple scab, we focused on the epiphytic phase of the development of the disease, germination and penetration of the conidia of *V. inaequalis* and tested if one of these events was affected by the treatment. This experiment was completed by an evaluation of the yeast effect on the scab severity. For the codling moth, as the first contact between the insect and the yeast occurs when the females land on the plant to lay eggs, we examined the yeast's effect on the quantity of eggs laid. Moreover, a field experiment allowed an evaluation of the yeast's effect on the fruit damage linked to the codling moth. Finally, to evaluate the effect on the fruit, we measured the main quality parameters.

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Materials and methods

Yeast preparation for all the following experiments

The yeast was bi-weekly plated out onto Potato Dextrose Agar (Oxoid, Basingstoke, UK) and incubated at 24°C. These plates were used to inoculate 100 mL Potato Dextrose Broth (Oxoid, Basingstoke, UK) in a 250-mL flask. These cultures were incubated at room temperature for 48 h on a rotary shaker (140 rpm).

Before treatment, the yeast suspension was separated from the growth medium through a double centrifugation of 20 min each ($5000 \times g$ at 4°C). Treatment consisted of a water suspension supplemented with Tween 80 (T80) also known as Polysorbate 80, that was sprayed until runoff with a hand pressure sprayer (Model DEA 2000, Davide & Luigi Volpi S.p.A., Casalromano, Italy). The cell concentration ranged from 5×10^6 to 5×10^7 cells/mL. It was determined by measuring the optical density (OD) of the suspension with a spectrophotometer at 450 nm using an OD-cell concentration standard curve. The same yeast concentration was used in all the trials that were carried out to evaluate the efficacy of the yeast in powdery mildew control (Mendelsohn et al. 2006; Alaphilippe et al. 2007).

Effect of the yeast on the germination of **V. inaequalis** *conidia*

Open-pollinated apple tree (cv. Golden Delicious) seedlings with four to five leaves, grown in the greenhouse (16 h of light and $80 \pm 10\%$ of relative humidity) were used for this experiment. The youngest opened leaf of each seedling was marked with a permanent marker.

A first experiment was conducted to provide information on the *V. inaequalis* inoculum concentration to use for testing the inhibition potential of the yeast for the scab conidia germination and germ tube elongation. Conidia concentrations ranging from 10^4 to 10^6 conidia/mL caused symptoms with conidiation on all the inoculated leaves, covering the whole leaf surface. We first tested the efficacy of the yeast suspension on the lowest conidia concentration needed to ensure good severity.

For the following experiments, the conidia (inoculum) concentration was evaluated with a Thomas–Kammer haemocytometer and adjusted by dilution to a range of $(10 \pm 1) \times 10^3$ conidia/mL. For each set of experiments, the seedlings were divided into three groups (12–18 plants per group, according to plant availability). Seedlings were treated with one of the following treatments: (i) distilled water, (ii) yeast suspension, or (iii) yeast suspension supplemented with 0.01% Tween 80. Approximately 3 h after treatment, once the leaves were dry, the plants were inoculated with *V. inaequalis* conidia following the method described by Gessler and Stumm (1984). The inoculated seedlings were placed above a tray full of water and the whole structure was covered by a polyethylene bag, in order to bring the humidity level close to 100%. The covered seedlings were kept in the dark at 18°C for a 48-h infection period. At 3, 5 and 7 days after this inoculation, a sample of four to six marked leaves per treatment were prepared for microscope observations (Silfverberg-Dilworth 2003). The number of non-germinated conidia and the number of germinated conidia with and without penetrating structures were counted under a light microscope (Olympus BH-2) in samples of 100 conidia. The whole experiment was replicated four times.

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Effect of the yeast on the scab severity and AUDPC value

Experiments were conducted on 3- or 4-year-old, 'Golden Delicious' apple trees. These trees were grown in 25-L containers in a black net house and were irrigated by a drip system.

The infection was made by spraying the trees with a suspension of 1000 conidia per mL. This suspension was prepared by immersing in water, scab infected apple leaves with conidiation. Yeast treatments were applied 3 days before infection, 1 day after infection and 1 week after infection. Water sprayed trees were used as control.

Scab severity (percent of diseased leaf area) was evaluated on four branches of each tree and averaged per tree. There were four tree replicates for each treatment. AUDPC (Area Under the Disease Progress Curve) values were calculated. Data in percentages were Arcsine-transformed before further analysis. Disease severity and AUDPC data were analysed using ANOVA and Fisher's protected LSD test. Standard errors (S.E.) of the means were calculated. The statistical analysis was done with JMP software (SAS Institute, Cary, NC, USA).

Effect of the yeast on codling moth oviposition

These experiments were conducted in Trentino, Italy, during the second flight of the codling moth, corresponding to the month of August in both 2005 and 2006 on 'Golden Delicious Smoothee' apple trees, 4-years-old, grown in 25-L containers. Fungicide treatments against powdery mildew and scab were made when required, alternating trifloxystrobin (Flint®, water dispersible granule (500 g/kg), Novartis Crop Protection AG, Basel, Switzerland) with Penconazol + Sulfur (Topas Combi®, 1.5 and 40%, respectively, wettable particles, Syngenta AG, Basel, Switzerland). No chemical treatment was made in the month preceding the experiments. The experiments were carried out in the open but in a covered area (under a polycarbonate roof, 2.5 m high), in order to ensure a light intensity similar to that found inside the

tree canopy of a commercial orchard. All time measurements were recorded as local clock-time and later converted to solar time. Solar time was determined by the delay for noon-time between the local clock-time and the local solar noon. The local solar noon is established in function of the local sunrise and sunset data (Armbruster and McCormick 1990). The yeast suspension was sprayed on the trees 24 h before the insect release. Each experiment consisted of eight treated and eight untreated trees. Each group of eight trees was composed of trees with different morphology: different numbers of fruits, branches, bourse shoot branches and leaves, but the groups of trees, treated and untreated for 2005 and 2006, were similar. For the 2005 season, the eight treated and non-treated trees were separated into two groups of four trees and treated under similar climatic conditions but with a 2-day interval.

Riedl and Loher (1980) observed that oviposition occurred towards the end of the day with a distinct peak before sunset. As most of the eggs are laid in the 5–6-h period preceding night fall, the insects, 18–20 gravid females (Andermatt Biocontrol AG Mass-Rearing, Grossdietwil, Switzerland), were released at 15:00 h solar time under white net cages (1 m long $\times$ 2.2 m high), each of which contained one apple tree. The oviposition period lasted until the following morning at 06:00 h solar time, after full sunrise. In the first group of experiments of 2005, the insects were purchased from INRA Le Magneraud (France). According to Levene's test for relative variation (Schultz 1985), the insects' origin (INRA Le Magneraud or Andermatt Biocontrol AG Mass-Rearing) did not affect the homogeneity of the variance ($F = 2.44$, $P = 0.17$) (STATISTICA, version 7.0, Statsoft Italia Srl, Vigonza, Italy). Eggs were counted on different tree tissues after the end of the release period. The examined tree tissues included: fruit, leaves from the corymb, leaves from the bourse shoot and 'other' leaves (other = all the other types of leaves). (The 'bourse' is a floral growth unit developing from a resting bud and composed of vegetative organs in its proximal part and floral organs in its distal part (Escobedo-Alvarez 1990).) Tree sections are presented in Figure 1, which is based on the description of Costes, Sinoquet, Kelner and Godin (2003).

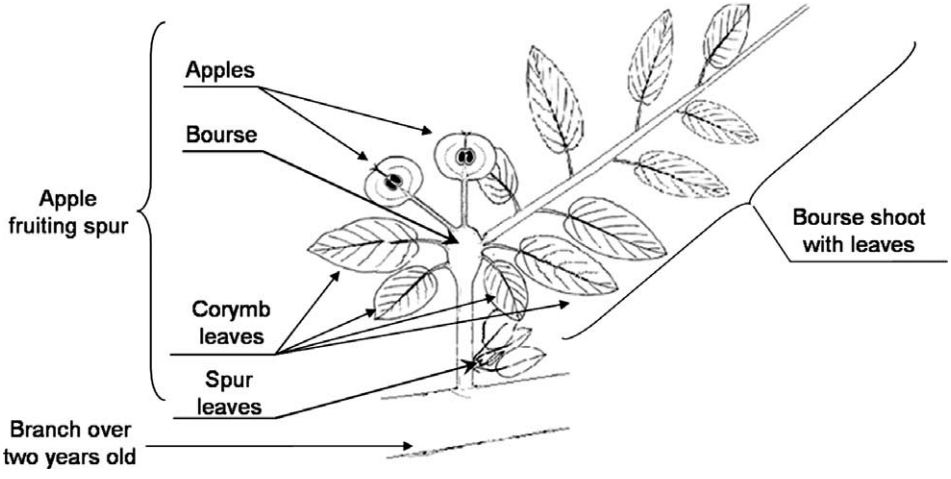

Figure 1. An apple fruiting spur with the localisation of the different tree sites and leaf types. The drawing is based on the description of Costes et al. (2003).

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The 'other' leaves were separated into two groups as follows: (i) leaves located less than 20 cm from fruits and (ii) leaves located more than 20 cm from fruit. This distinction was made because around 95% of codling moth eggs are usually laid on fruit or leaves located less than 20 cm from fruit (Mattedi and Zelger 2006).

The effects of the yeast on fruit damage caused by codling moth (field trial 2006)

The orchard was divided into 32 blocks of 30, cv. Golden Delicious trees, each distributed over two rows that were planted 2 m apart. Every two rows of 'Golden Delicious', a row of 'Red Gala' was planted. The trees were 15-years-old and about 3 m high. From among these blocks, we selected eight blocks with the same pest pressure (around 12–14% of damaged fruits during the first codling moth flight), in which we stopped all insecticide treatment during the second flight of the codling moth, from mid-July through to the end of August (2 weeks before apple harvest). Four of these blocks were treated with the yeast suspension twice a week (for 7 weeks), according to the weather conditions. The other four blocks served as an untreated control. Each block of 30 trees was sprayed with 18 L of an aqueous suspension of yeast (10^7 cells/mL) supplemented with Tween 80. The treatment was applied in the late afternoon (16:00 h solar time), in order to avoid immediate exposure of the yeast to very strong direct sunlight. Fungicide treatments were applied following the traditional calendar of treatments (Table 1). A preliminary experiment showed that the yeast was not affected by applications of these commonly used fungicides: copper hydroxide, Kocide 2000 dry flowable (35%), Dupont, Wilmington, DE, USA; Suffer, Thiovit 80 micronised wettable with spherical particles (80%), Syngenta AG, Basel, Switzerland; Triadimenol, Bayfidan, Emulsifiable Concentrate (250 g/L), Bayer CropScience AG, Monheim Germany; Hexaconazole, Anvil 5SC, solution concentrate (50 g/L), Novartis Crop protection AG, Basel, Switzerland; Tebuconazole, Folicur 80 WG emulsion (251.2 g/L), Bayer CropScience AG; pyrimethanil, Mythos, Suspension concentrate (300 g/L), Hoechst Schering AgrEvo GmbH, Berlin, Germany; Kresoxim-methyl, Stroby WG, water dispersible granule (500 g/kg), BASF, Ludwigshafen, Germany; Mancozeb & Famoxadon, Clipman, Water dispersible granules (62.5 and 6.25%, respectively), Dupont; Fluazinam, Ohayo, Suspension concentrate (500 g/L), Syngenta; Captan, Merpan 80 WG, micronised wettable with spherical particles (80%), Makhteshim Chemical Works LTD, Beer Sheva, Israel.

Fruits were collected at three different times during the second flight of the codling moth i.e. 3, 6 and 8 weeks after the start of the experiment, the last one being 1 day

Table 1. Treatments sprayed in the tested orchard just before and during the field experiment period in 2006, calendar of application and product details.

Date	Diseases	Active ingredient	Commercial name	Dose (mL/hL or g/hL)*
15 July	Scab	Dithianon	Delan	20
	Powdery mildew	Triadimenol	Bayfidan 5 wp	60
2 August	Apple 'bitter pit'	Calcium chloride	Neobit	400
19 August	For conservation	Ziram	Triscabol	150

*13 hL are sprayed per hectare.

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before harvest. For each collection, 250 randomly selected fruits per treatment and block were examined. In order to evaluate the damage caused to fruit by the codling moth, we counted the number of fruit penetrations and classified them according to severity using a system based on the description of Ballard, Ellis and Payne (2000), i.e. fresh (damage by early first instar larvae where the apple skin has healed, leaving a red 'sting' or small pits of browned tissue with no exterior frass), blocked (holes with or without frass) and a tunnel in the apple, without the presence of the larva and active (larger and messy holes with visible frass and a tunnel in the apple; larvae may be present). Frass consists of debris or excrement produced by insects.

Effect of the yeast on fruit quality (field trial 2006)

In the same orchard described above, we sampled 30 ripe apples from each of the eight blocks (four treated and four untreated). Apple quality parameters were measured with an automated instrument, Pimprenelle (Setop Giraud-Technologie, Cavaillon, France) located at the Research Unit for Fruit Conservation of the Istituto Agrario di San Michele All'Adige (Italy). The examined parameters were sugar content (tested with a refractometer), firmness (kg/cm^2), acidity (NAOH/mL), juiciness and the percentage of dry substances, as well as fruit size and weight.

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Statistical analysis

When not otherwise specified, the analyses were performed using the statistical software STATISTICA, version 7.0 (Statsoft Italia Srl, Vigonza, Italy). The percentages of germinated conidia (with or without penetrating structures) as well as the percentage of penetrated conidia were compared using the Student's *t*-test. Results of all insect experiments and the fruit quality parameters were compared using the Mann–Whitney *U*-test. For convenience, data are presented in the text as mean $\pm$ standard error (S.E.) of the mean.

Results

The effect of the yeast on the germination of V. inaequalis *conidia*

At 3, 5, and 7 days after yeast treatment, the yeast spray mixture supplemented or not with Tween 80 had no effect on the germination of *V. inaequalis* conidia, or on their ability to penetrate the plant tissue (Student's *t*-test, $P > 0.1$) (Table 2).

For all treatments, the quantity of conidia with a penetration structure was higher 7 days after treatment, compared to 1 day after treatment. The percentage of germinated conidia, with and without penetrating structures, on untreated leaves 7 days after the inoculation was $55 \pm 8\%$. Germination and penetration were not affected by yeast alone or supplemented with Tween 80 (50 ± 8 and $53 \pm 6\%$ of germinated conidia, respectively). Figure 2 shows one germinated conidium on an apple leaf surface with the germ tube and appressorium surrounded by yeast cells: the yeast cells did not affect the conidial germination.

Table 2. Effects of the yeast spray mixtures supplemented or not with Tween 80 (T80) on the germination and leaf penetration of *Venturia inaequalis* conidia according to the time after inoculation.

	Germination without penetration			Penetration		
Time	Water	Y16	Y16+T80	Water	Y16	Y16+T80
3	36±4.5 ***a***	27±2.9 ***a***	30±2.3 ***a***	5±1 ***a***	5±0.9 ***a***	2±0.4 ***a***
5	27±4.0 ***ab***	26±2.8 ***ab***	26±4.0 ***ab***	11±2 ***a***	7±1.5 ***a***	4±0.9 ***a***
7	23±2.8 ***ab***	20±3.5 ***ab***	14±1.9 ***b***	32±5 ***b***	30±4.9 ***b***	39±3.7 ***b***

The evaluation of the percentage of germinated conidia and fungal penetrations (primary stroma formation) per treatment and observation time was counted under a light microscope (Olympus BH-2) in samples of 100 conidia on four to six leaves. Different letter indicates significant differences according to the LSD Fisher test.

Effect of the yeast on the scab severity and AUDPC value

Upon visual observation of seedlings inoculated with apple scab, we found no difference in scab symptoms between treated and untreated seedlings. Indeed, 12 days after inoculation, susceptible leaves of both treated and untreated seedlings were all covered by conidiation of *V. inaequalis*.

The disease progression as shown in Figure 3 was lower on the yeast treated plant when compared to the water control trees. Infections caused by *V. inaequalis* were significantly less severe on the yeast treated trees than on the control trees. Scab severity reached 32.5±1.9% on the water control and 16.2±2.2% on the Y16-treated plants, which indicates a 50% decrease in disease severity. This difference, as well the difference 24 days after infection, was significant according to Fisher's protected LSD test ($P<0.05$).

The AUDPC values (Table 3) for all four experiments were significantly lower on the last day of the experiment (17, 24 and 36 days after infection) on the yeast treated

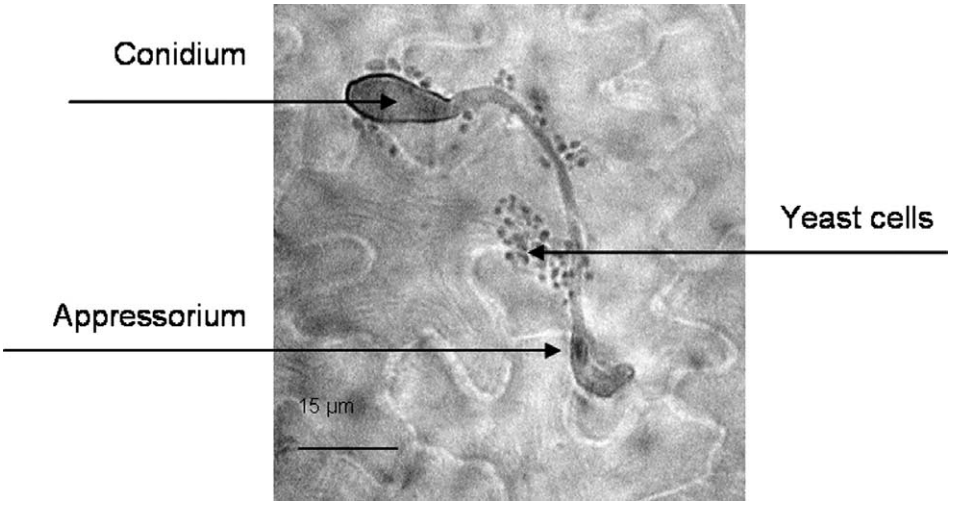

Figure 2. Light micrograph of a germinated conidium of *V. inaequalis* with the germ tube and the appressorium on apple leaf surface. Yeast cells are visible around the germ tube.

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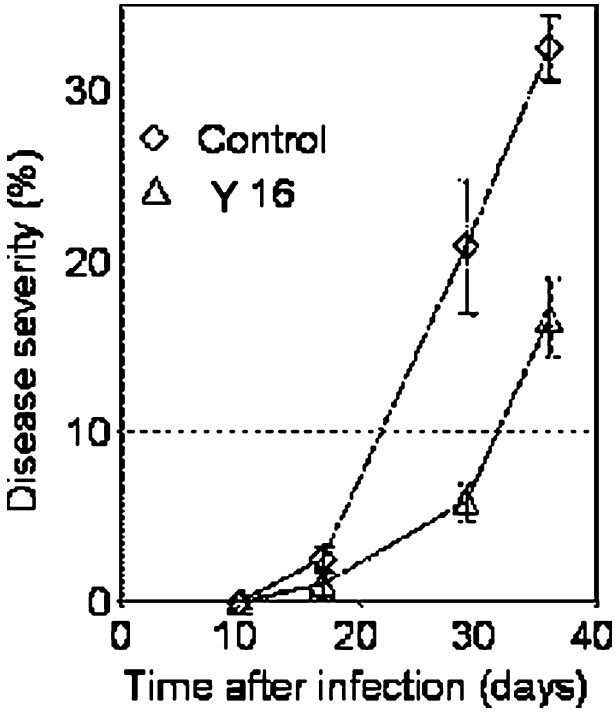

Figure 3. Effect of the yeast isolate Y16 on scab disease severity (% of diseased leaf area) according to the time after the *Venturia inaequalis* conidia inoculation. Bars represent the standard errors (S.E.) of each mean.

trees, compared to the water control trees. The Y16 decreased the AUDPC by up to 79.8% (experiment 2).

Effect of the yeast on codling moth oviposition

In 2005, the total number of eggs laid per tree was significantly higher on the trees sprayed with the yeast suspension (107 ± 17) than on the untreated trees (52 ± 15).

Table 3. Effect of the yeast isolate Y16 on scab (*Venturia inaequalis*) in experiments with apple trees.

	Experiment number			
Treatment	1	2	3	4
Water control	335.4 ± 16.1	109.7 ± 5.1	242.1 ± 12.3	384.0 ± 18.4
Y16	123.9 ± 5.15	22.2 ± 3.6	129.5 ± 6.2	250.9 ± 12.5
Disease reduction (%)	63.1 *[T]	79.8 *	46.5 *	34.7 *
Duration of experiment (days)	36	24	24	17

Disease severity as Area Under the Disease Progress Curve (AUDPC) in % *days at the last day of experiment. [T] Treatments marked with an * are significantly different from the respective untreated control according to Fisher's protected LSD test at $P = 0.05$.

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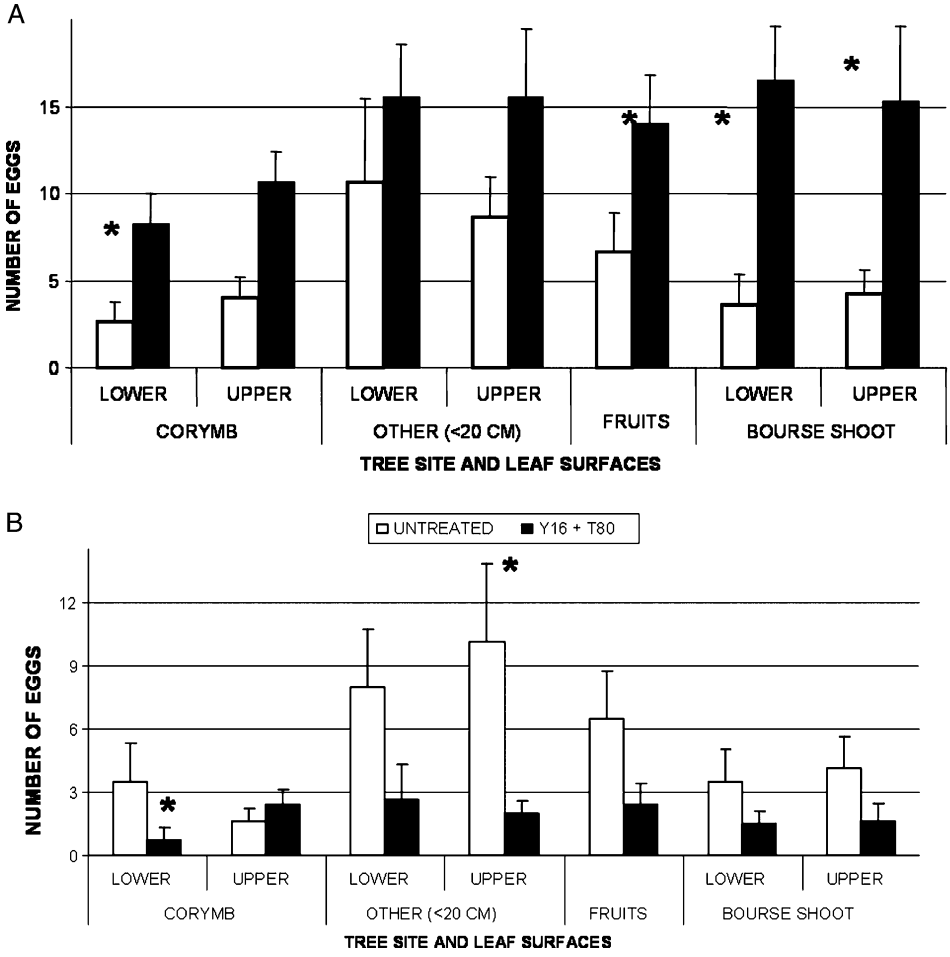

Figure 4. Effect of the yeast treatment on the quantity of eggs laid by *Cydia pomonella* females. The experiments were carried out in the orchard, but in a covered area. The evaluation of the egg quantity was made according to the tree site (spot on the tree where tissue was sampled) and the leaf side as compared with egg laying patterns on untreated trees. Bars represent the standard errors (S.E.) of each mean; (*) indicates significant differences in egg quantities according to the Mann–Whitney *U*-test ($P < 0.05$). This figure shows the mean values of eight replicates. Data from (A) 2005 and (B) 2006.

This difference in the quantity of laid eggs was consistent across all examined tree sites and on both sides of the leaves (Figure 4A). Moreover, the egg distribution within the tree was affected by the yeast treatment. The effect was significant for most of the tree sites closest to the fruit, which means that, on the treated trees, there were many eggs on the fruits and on the leaves closest to the fruit as shown in Table 4. Moreover, the mean quantity of eggs laid per fruit for treated trees (1.97 ± 0.45) was more than two times higher than for untreated fruits (0.91 ± 0.38) (Mann–Whitney *U*-test, $P = 0.09$).

In 2006, the effect of the yeast was different; the treatment was associated with a decrease in the number of eggs laid (Figure 4B and Table 4). The average number of eggs laid per untreated tree was 46.1 ± 5.4 and the number of eggs was 14.4 ± 1.3 on

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Table 4. Effect of the yeast treatment according to the year of experiment (2005 and 2006) on *C. pomonella* egg laying on different parts of the apple trees as compared with egg laying patterns on untreated trees.

	Untreated			Y16+T80		
Organ type	2005	2006	*P*	2005	2006	*P*
Total prox*	40.4±13.6	37.4±13.6	0.85	95.6±15.9	13.3±3.4	0.01
Total (eggs laid per tree)	52±15	46.1±5.4	0.77	107±17	14.4±1.3	0.01
Eggs laid per fruit	0.91±0.38	0.75±0.39	1	1.97±0.45	0.26±0.15	0.02

P value according to the Mann–Whitney *U*-test is indicated. Average number of eggs laid per tree and per organ within each tree (eight trees per mean value). Experiments were carried out in the open, but under a polycarbonate roof. * 'Total prox' is the total number of eggs on fruits and on leaves located less than 20 cm from fruit.

the yeast-treated trees. This difference in egg numbers was significant on the lower parts of the corymb leaves (Mann–Whitney *U*-test, $P=0.03$) and the upper parts of the other leaves (Mann–Whitney *U*-test, $P=0.02$). As shown in Table 4, there was no difference, according to Mann–Whitney *U*-test ($P=0.29$), between the quantity of eggs laid per treated fruit (0.75±0.39) and per untreated fruit (0.26±0.15). It also appeared that for treated trees the eggs distribution within the tree was not linked to the tree sites: the quantity of eggs was low on all tree sites (one to two eggs laid in average per site).

In 2005, the average quantity of eggs laid on the fruits and on leaves less than 20 cm from the fruit (called 'total prox' in Table 4) of treated plants was more than double that on the untreated plants (Mann–Whitney *U*-test, $P=0.04$). In 2006, the average egg quantity on the sites close to the fruit ('total prox') was 2.4 times lower for the treated trees than for the untreated trees (Mann–Whitney *U*-test, $P=0.06$).

As shown in Table 4, the quantity of eggs laid on the control trees in 2005 was similar to the amount laid in 2006 (Mann–Whitney *U*-test, $P=0.77$). Moreover, the egg distribution within the plant was identical both years. However, the effect of the yeast 1 day after treatment on egg laying was significantly different between the 2 years (Mann–Whitney's *U*-test; $P=0.01$). Applications of the yeast had a different effect on *C. pomonella* egg laying in 2005 and 2006.

Table 5. Effect of the treatment (Y16+T80) on fruit damage caused by *C. pomonella*: percentage of fruits with fresh, active or blocked lesions (mean±S.E.) caused by the codling moth at different dates; the last date corresponds to the day before harvest.

	Fresh lesions		Active lesions		Blocked lesions	
	Control	Y16+T80	Control	Y16+T80	Control	Y16+T80
9 Aug. 2007	1.9±0.5 ***a***	2.0±0.5 ***a***	0.5±0.1 ***a***	1.8±0.4 ***a***	0±0 ***a***	0.3±0.1 ***a***
1 Sept. 2007	5.3±0.6 ***b***	4.6±1.7 ***b***	4.4±1.4 ***a***	2.2±0.5 ***a***	1.2±1.1 ***ab***	2.2±1.3 ***ab***
13 Sept. 2007	0.6±0.2 ***a***	0.4±0.2 ***a***	11.1±3.1 ***b***	13.6±1.5 ***b***	4.6±1.1 ***c***	3.5±0.6 ***bc***

Experiment conducted on four blocks per treatment, evaluation of the percentage of damaged fruits and of the damage type on 250 randomly selected fruits per block. Different letters indicate significant differences according to the LSD Fisher test.

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Effect of the yeast on fruit damage caused by the codling moth (year 2006)

In both treated and untreated blocks, fruit damage increased over the course of the season. In the untreated block, on 9 August, 2 weeks after the first yeast spray, 2% of the fruit was considered damaged as compared to 16% of the fruits on 13 September, 1 day before harvest. In the treated block, damage increased from 4% on 9 August to 18% on the day before harvest. Among the damaged fruit, the proportions of blocked, fresh and active lesions were similar in the treated and untreated blocks (Table 5). On 13 September, the proportion of fresh lesion was very low, which corresponded with the end of the active period for egg laying of the codling moth in the Trentino area (Italy).

The treatment affected neither the proportion of fruit damaged by the codling moth nor the type of damage. This trend was consistent across all three evaluation dates 9 August, 1 September and 13 September.

Effect of the yeast on fruit quality (field trial)

The treatment (Y16+T80) did not affect any of the main apple fruit quality parameters (Figure 5) according to the Mann–Whitney *U*-test (i.e. sugar content (refractometric method) $P=0.21$, firmness (kg/cm^2) $P=0.12$, acidity (NAOH/mL) $P=0.77$, juiciness $P=0.15$ and the percentage of dry substances $P=0.25$).

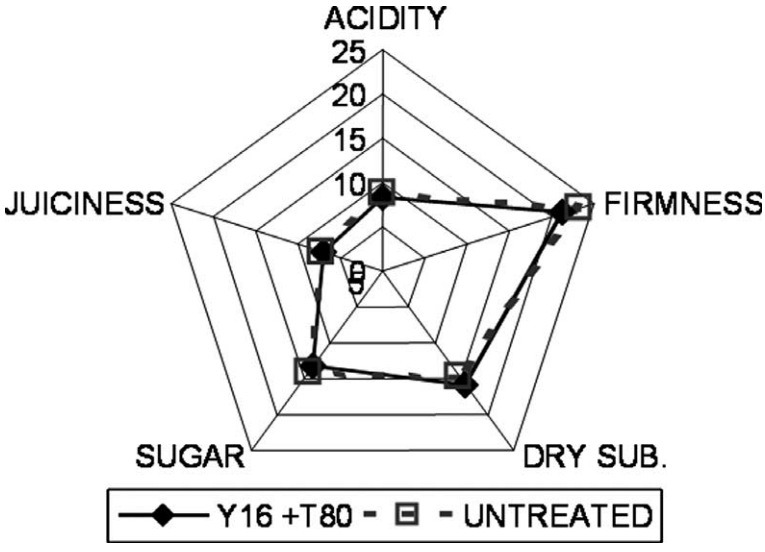

Figure 5. Effect of the treatment (Y16+T80) on the main apple fruit quality parameters (i.e. sugar content (refractometric method), firmness (kg/cm^2), acidity (NAOH/mL), juiciness and the percentage of dry substances). Spider plots of the quality parameters measured by Pimprenelle on four groups of 30 'Golden Delicious' apples treated with the yeast suspension, as compared with four groups of 30 untreated control apples to characterise the yeast effect on the fruit quality.

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Discussion

In this work, we examined the effects of the introduction of Y16 to apple tree, on the non-target organisms apple scab and the codling moth, and host plant fruit production.

It was found that the yeast did not affect the germination or penetration of *V. inaequalis* conidia when applied 3 h before pathogen inoculation. Moreover, as shown in Figure 2, the conidia and the germ tube were not affected by the presence of the yeast. However, regarding the scab severity and AUDPC, the yeast, when applied 3 days before pathogen inoculation, reduced the disease severity and progression. The effect of epiphytic microorganisms on scab is seldom reported. This may be due to the fact that the epiphytic phase of scab development is very short and independent of nutrient availability (MacHardy 1996); thus, competition for nutrients as a strategy for scab suppression is not reliable. Other mechanisms such as the induction of plant defence mechanisms, could explain the observed effect of the yeast on the pathogen severity, especially because the treatment was made 3 days before pathogen inoculation. Moreover, the conidia level used in the observation of the conidia germination and penetration may have been too high for a biocontrol agent to affect the disease. Indeed, less conidia were needed to induce the disease onset on whole plants and thus, allow the yeast to affect the *V. inaequalis* development.

Similarly, negative effects of the use of biocontrol agents on fruit yield and quality are seldom reported (Swadling and Jeffries 1996), but as such negative effects were already observed, we found it appropriate to look at the potential effects, of regular spraying of a biocontrol agent, on the main fruit quality parameters. In the present work, no effect of the yeast Y16 on the main fruit characteristics was found.

Concerning the effect of the yeast on the codling moth egg laying behaviour, we found that 24 h after application, the yeast significantly affected *C. pomonella* egg laying. However, the effect differed between the two observation years. In 2005, the quantity of eggs laid per yeast-treated tree was higher when compared to the untreated trees, whereas in 2006, it was lower on yeast-treated trees than on the untreated trees.

In 2005, the quantity of eggs laid was especially high on the sites close to the fruit, which corresponds to a higher probability for hatching larvae to successfully reach and penetrate the fruits and as a consequence to damage them. As observed by Geier (1963), approximately one-third of the eggs laid results in successfully established larvae. So, the fact that the quantity of eggs laid per fruit and on tree sites close to the fruit was higher on treated trees compared to untreated trees confirmed a risk increase for fruit damage linked to the treatment. Whereas the mean quantity of eggs on the fruits give an idea of the preference of the insect for this organ compared to the different leaf types.

In 2006, the laid egg quantity tended to be lower on the treated trees compared to untreated trees, but the mean quantity of eggs laid per fruit was not affected by the yeast treatment, so the risk of fruit damage was not modified by the yeast applications. In the field experiment conducted in 2006, we did not find any effect of the yeast applications on the amount of fruit damage caused by this moth, or on the type of damage. This experiment was conducted over a 2-month period and supports the results obtained in the semi-natural conditions of the egg laying experiment carried out in 2006.

The good stability of the mean value of eggs laid per females on the control trees in both seasons (Table 4), as well as the relatively low variability prove that the protocol used to test the yeast effect on egg laying is valid. However, the mean number of eggs laid per female (2.6 in 2005 and 2.3 in 2006) on the control trees is low compared with the expected potential for a single codling moth female to lay 15 to 25 eggs (Knight 1997). This phenomenon could be partly explained by the high quantity of non responding females. Indeed as the insects were mass-reared under optimally controlled conditions, the change in climatic conditions could disturb their egg laying behaviour (Kuhrt, Samietz and Dorn 2006). Moreover, mass-reared insects are known to lose sensitivity to host cues (Mattiacci, Hutter, Schoch, Scascighini and Dorn 2000; Gandolfi, Mattiacci and Dorn 2003). Both of these factors, environmental change and lost of sensitivity could explain the low quantity of eggs laid per female.

The opposing effects found between the two experimental seasons could be explained by a difference of activity of the yeast between the two seasons of experiments 2005 and 2006. In 2006, the climatic conditions were different compared to 2005. Indeed the mean temperature was 4°C lower than in 2005, and the relative humidity was around 15% higher. Since microorganism establishment and survival is dependant on climatic conditions (Burges 1998; Jones and Burges 1998) such as temperature and humidity, we hypothesise that the egg laying varied between the 2 years of the experiment, because the plant–yeast interaction was modified by the different climatic conditions. Martin et al. (1993) already suggested that the influence of microorganisms on insect behaviour may be different according to the microorganism abundance and to biotic and abiotic factors of the environment. Further studies to analyse the direct effect of the microorganism on egg laying behaviour, independent of the plant interaction, are currently in progress. These experiments will allow us to evaluate the yeast effect on egg laying at a known concentration and known environmental conditions.

Studies of the mechanisms of tritrophic plant–yeast–insect interactions are complex and must take into account insect behaviour, which is often poorly understood. We can suppose that an epiphytic yeast could affect the odour and/or taste of the plant and the contact between the insect and its host plant (Darriet et al. 2002). These changes could, in turn, influence the insect's ability to select and recognise the host plant, egg survival and larval survival and orientation (Stadler 2002). Preliminary experiments showed induced resistance as possible mode of action of Y16. It has been already found, that an inducer of systemic resistance of apple trees against *Erwinia amylovora*, the Acibenzolar-*S*-Methyl (ASM), affected the codling moth egg laying. This effect was correlated with a change of the apple leaf surface primary metabolites (Derridj and Borges 2006). Moreover, it has been established that a change in soluble carbohydrates and sugar alcohols present on the leaf surface could partially explain a change in codling moth egg laying (Lombarkia and Derridj 2002) and it is known that epiphytic microorganisms consume the nutrients present on the leaf surface (Andrews 1992; Mercier and Lindow 2000). Therefore, we could expect a change in the leaf surface composition by the introduction of the epiphytic yeast and as a consequence a modification of the insect egg laying behaviour. Analyses of the phylloplane composition are currently in progress. While it is possible that leaf surface characteristics are perceived by contact chemoreception, olfaction may also be involved (Woodhead and Chapman 1986).

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Conclusion

This yeast could potentially be used as a biocontrol agent (BCA) against apple powdery mildew. It did not modify the principal fruit characteristics or quality, nor favour the development of *V. inaequalis*. Indeed, at low levels of *V. inaequalis* conidia inoculation, it even significantly decreased disease severity. This effect is interesting as the treatments against both diseases are made at the same period. In addition, this microorganism is not affected by the application of commonly used fungicides. Before developing this yeast for use as a BCA in commercial apple orchards, we should understand its effect on the codling moth, since this insect is one of the most important pests of European apple trees. In this study, we showed that the Y16 had an effect on the codling moth egg laying 24 h after application, but this effect differed according to changing environmental conditions. This effect was studied on the second flight period of the codling moth, corresponding to the period in which the last treatments against the powdery mildew are made. In 2005, the yeast treatment strongly increased the quantity of eggs laid on the treated trees, especially on the sites close to the fruits leading to a higher risk of yield loss. The possibility of negative economical consequences from the use of this potential control agent could not be eliminated in this study. However, this effect was neither confirmed by the second trial conducted the following year in semi-natural conditions nor by the field experiment. In the field, fruit damage in the treated and untreated plots were similar. Thus, it could be interesting to follow the populations of insect pests (not only *Cydia pomonella*) during the first years of the widespread use of this BCA. This work demonstrates that more importance should be given to studying the consequences of the widespread introduction of new biocontrol agents, on non-targeted pests and diseases.

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