ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2012, Vol 19, No 4, 631-636
www.aaem.pl

Health effects of selected microbiological control agents. A 3-year follow-up study

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Baelum J, Larsen P, Doekes G, Sigsgaard T. Health effects of selected microbiological control agents. A 3-year follow-up study. Ann Agric Environ Med. 2012; 19(4): 631-636.

Abstract

Introduction and objectives: Microbiological control agents (MBCA) are widely used in greenhouses, replacing chemical pesticides. The presented study aims to describe health effects of exposure to three types commonly used: *Bacillus thuringiensis, Verticillium lecanii,* and *Trichoderma harzenianum* covering seven different products in greenhouse workers with emphasis on sensitization and respiratory effects.

Methods: 579 persons aged 17-67 years culturing ornamental flowers were included. They were followed for three years with annual examinations including interview about exposure and symptoms, lung function, including bronchial (histamine) challenge test, and blood samples. Direct and indirect exposure for each person and year was estimated by information from respondents and employers. IgE in serum against the 7 products of MCBA was analyzed using an enzyme immunoassay technique.

Results: 65%, 40%, and 78% were exposed to *B. thuringiensis, V. lecanii,* and *T. harzenianum,* respectively, while 6, 3 and 3% were handling the products. IgE against *B. thuringiensis* was seen in 53% of the samples and with prevalence rate ratios among exposed increasing from 1.20 (CI95%:1.01-1.42) to 1.43 (CI95%:1.09-1.87) over the 3-year period. There was no relation between exposure to any MBCA and neither prevalence nor incidence of respiratory symptoms and there was no effect on lung function or bronchial responsiveness.

Conclusions: Use of *B. thuringiensis* in greenhouses may give rise to sensitization while no effect on the occurrence of respiratory symptoms or lung function was observed. The persons had a relatively long exposure. Therefore, a healthy worker effect may have influenced the results.

Key words

Bacillus thuringiensis, *Trichoderma harzenianum*, *Verticillium lecanii*, sensitization, health effects

INTRODUCTION

During the last decade the use of pesticides for plant protection, to a large extent, has been replaced or supplemented by the use of biological pesticides in greenhouses. These biological agents have either specific insect toxicity or are competing with plant pathogens. Compared to the chemical pesticides these agents have been considered safe for mammals, but spreading large amounts of bacteria in a closed environment as a greenhouse may imply risk for health by the sheer amount of organic material the workers which the workers may be exposed. Three different types of microbiological control agents (MBCA) have been extensively used in Danish indoor production of ornamental plants and included in the presented study.

Bacillus thuringiensis species have been extensively used as insecticides in greenhouses, on outdoor crops, and in woodlands. The insecticidal active CRY- toxin is highly specific for selected insects and the main concern has been the close familiarity with other human pathogenic species *B. cereus* and *B. Anthracis* [1]. However, gastrointestinal colonization and infection with MBCA have been sparsely reported [2, 3]. With regard to allergy and respiratory effects, human reports are also sparse. Bernstein et al. reported some increase in skin prick test response, as well as IgE and IgG levels in a group of high exposed workers 1-4 months after spraying with products of *B. thuringiensis*, with a decline in both IgE and IgG from 1-4 months, while a less exposed group only handling the treated products showed no response. No respiratory or skin symptoms were related to *B. thuringiensis* exposure [4]. In our preliminary study, we did find some sensitization to products of *B. thuringiensis*, but no relation to its use in greenhouses or symptoms [5].

Verticillium lecanii (Lecanicillium lecanii) (products Mycotal® or Vertalec®) is an insecticidal fungus exhibiting its effect by overgrowing plant pathogenic larvae. Despite being widely used only limited reports of human exposure or effects have been published. Eaton et al. made a survey on the research centres developing the agents [6]. They found a limited number (5%) of positive skin prick tests without relation to exposure and no relation to symptoms or lung function. A related plant pathogenic fungus *V. albo-atrum* has been identified as a causal agent in relation to an asthma outbreak in tomato growers [7].

Trichoderma harzeniani has been used against pathogenic fungi, especially grey mould (*Botrytis cinerea*) acitng by competition. The fungus, naturally occurring in several

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Received: 14 February 2012; accepted: 24 September 2012

environments, has been shown as an opportunistic infection in immuno- compromized patients, but none in healthy persons [8]. A recent study has indicated *T. harzianum* as being an important airborne allergen, measurable in ambient air, at least in India, showing a sensitization rate of 27% in a group of patients with allergic rhinitis [9].

Asthma has been shown to be prevalent among greenhouse workers and several agents as specific plants, moulds, and the spider mite (*Tetranychus urticae*) have been identified as causes of asthma [10, 11]. In Denmark, about 6.000 persons are employed in greenhouses, of these the majority cultivating ornamental plants. The use of chemical pesticides has been steadily declining and MBCA's have been introduced since the 1980's.

The presented study, set up to investigate the health effects of MCBAs and a survey of greenhouse companies on the island of Funen in 1996, showed that products of *B. thuringiensis, T. harzianum,* and *V. lecanii* were the most prevalent agents used.

A 3-year follow up-study was designed to study the sensitization and health effects prospectively in a selection of the greenhouse firms.

MATERIAL AND METHODS

Design. During 1997-2001, greenhouse workers employed at 31 enterprises culturing ornamental flowers were followed. The enterprises were selected from respondents to a survey on the use of biological pest control carried out in 1996 in the greenhouse trade on the island of Funen, Denmark. Among 220 registered enterprises employing about 3,000 persons, 193 responded. Among these we contacted a number of enterprises employing at least 10 persons and reporting a spectrum of the use of the most abundant types of microbiological pesticides; 31 enterprises employing 773 persons were willing to participate, while a few declined for various reasons.

The follow-up study was carried out in 4 annual examinations (run 0-3) mainly during the winter seasons (run 0 – May 1997-Jan 1998, run 1 – December 1998- March 1999, run 2 – January-March 2000, run 3 – January-March 2001).

Study population. All persons employed were invited to participate in an introductory meeting. Of the total of 773 employed, 456 persons (59%) were willing to participate in the first examination round (run 0). The persons not willing to participate mainly gave fear of blood sampling as the reason. Besides, a number of persons were sick or had other reason to be off work on the particular days.

In run 1 (1998-9), the cohort was supplemented by 123 additional persons, who were either newly employed in the intervening period or not available in run 0. In total, the cohort consisted of 579 persons, 184 males and 395 females. Background data of the population is shown in Table 1.

The schedule of the study is shown in Figure 1. There was a loss of 140 (31%) persons between run 0 and run 1. Thirteen of these, however, participated in the next runs. Among the 123 persons included in run 1, 70% participated in run 2. Of the persons participating twice, 85% participated in the third, while 99% of those having participated three times were also included in the fourth. In total, 262 persons were followed for 3 years, 342 were followed for at least 2 years, while 402 were followed for at least 1 year. This gave 1,585 single observations and 1,006 observations of incidence data covering 1,146 person years.

Table 1. Key variables for persons in the study. The values of age, seniority smoking habits, and positive prick tests are at inclusion in the study

		Run 0	Run 1	Run 2	Run 3
	Males	148 (32 %)	148 (34 %)	123 (35 %)	116 (34 %)
	Females	308 (68 %)	291 (66 %)	227 (65 %)	224 (66 %)
	Total	456	439	350	340
Age (years) Mean and range	Males	34.8 (17-60)	34.7 (17-67)	35.8 (19-67)	36.3 (20-67)
	Females	35.7 (16-59)	35.9 (19-61)	36.8 (19-59)	36.9 (19-59)
Seniority in the trade Mean and range	Males	14.9 (0-45)	14.5 (0-53)	16.0 (0-53)	16.8 (0-53)
	Females	8.5 (0-38)	8.7 (0-38)	9.4 (0-38)	9.5 (0-38)
Seniority at the actual workplace Mean and range	Males	8.4 (0-39)	8.0 (0-39)	8.8 (0-39)	9.3 (0-39)
	Females	5.7 (0-33)	5.5 (0-33)	6.3 (0-32)	6.5 (0-33)
Positive pricktest Number (percentage)	Males	34 (23 %)	38 (26 %)	29 (24 %)	28 (24 %)
	Females	49 (16 %)	50 (17 %)	43 (19 %)	42 (19 %)
Atopy in the family Number (percentage)	Males	47 (32 %)	42 (28 %)	32 (26 %)	32 (28 %)
	Females	97 (31 %)	96 (33 %)	74 (33 %)	73 (33 %)

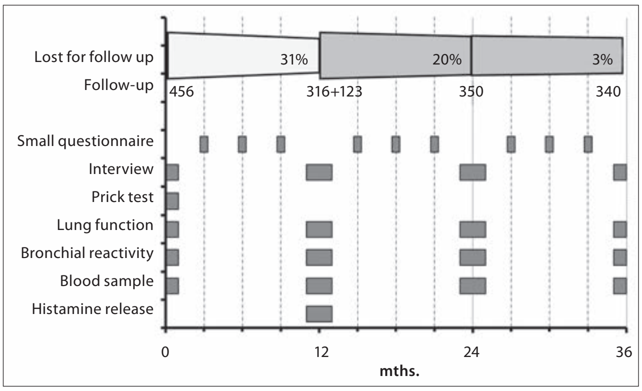

Figure 1. Design of the 3-year follow-up. The first rows show the flow of persons with the loss for follow up. The following rows show the scheduling of measurements

After runs 1-3, dropouts were contacted and asked to fill in a questionnaire about the cause of dropout. Out of 239 contacted 181 (75%) responded, while 2 persons were deceased. The main cause of dropout was change of company, trade, or temporary leave.

Measurements. In each run the persons were invited to participate at the workplace. The persons were given a self-administered questionnaire about the work conditions and a personal structured interview about health.

Lung function was measured using a dry bellow spirometer (Vitalograph® model R, Buckingham, UK) in accordance with the American Thoracic Society guidelines. The best values of FVC and FEV_1 were recorded, standardized in relation to predicted values calculated using the reference values supplied by Danish Society of Lung Physicians for persons above the age of 30 years [12]. Reference values for persons below the age of 30 were taken from a cohort of young Danish rural inhabitants [13].

Bronchial responsiveness was tested using histamine chloride challenge tests as described by Yan et al. using calibrated De Vilbiss No. 40 nebulizer (Somerset, PA, USA) [14]. The maximal cumulated dose was 1.23 mg in 1997-98 and 0.97 mg in 1998-99. The slope in decrease in FEV_1 in response to the logarithm of the cumulated dose of histamine was calculated and for persons responding with a more than 10 and 20%, decrease in FEV_1, respectively, PD_{10} and PD_{20} were calculated.

A standard prick test was set at the person's first participation including birch, grass, mugwort, horse, dog, cat, the house dust mites *Dermatophagoides pteronysious* and *D. Farinii,* as well as the fungi *Alternaria* and *Cladosporium*. A weal of 3 mm or more was classified as positive. Prick tests were also made for *T. harzianum* and *V. lecanii.* Prick test against *B. thuringiensis* could not be approved for use by the Danish Medicines Agency because of a possible content of milk products.

A venous blood sample was drawn and sera from the participants in each run were analysed for total IgE and specific IgE against the following products: Bactimos®, Dipel®, Vectobac® (*B. thuringiensis*), Supresivit®, Binab® (*T. harzianum*), Mycotal®, and Vertalec® (*V. lecanii*). The method of analysis has been published separately[5]. Antibodies against the 3 main components B. thuringiensis, *T. harzianum*, and *V. lecanii* were calculated as the sum of IgEs of the products. The limit of detection was set at 0.025 OD units.

The symptoms in the health interview were the lung symptoms cough, cough with phlegm, chest tightness, and wheezing. Upper airway symptoms were running and stuffed nose. Itching in the eyes, dermal rash, and seizures in accordance with alveolitis symptoms were also recorded. Each group of symptoms consisted of occurrence of the symptom within the last 12 months. This was supplemented with symptoms at work, in relation to specific activities at or without work, especially the handling of MBCAs. Finally, the frequency of the symptom was estimated (daily, more than once a week, more than once a month, or more seldom). Additionally, the respondents were asked about the occurrence of asthma within the last 3 months, and chronic bronchitis defined as cough and phlegm in more than 3winter months.

Familiar disposition was estimated by asking about asthma in either parent or in 1 or more siblings.

Exposure. According to the information from the greenhouse owners the products of the 4 types of Biological Control Agents (MBCA) used were: Bactimos®, Vectobac® (B*acillus thuringiensis* var *Israeliensis*) Dipel®, *Bacillus thuringiensis* var K*urstakii*), Supresivit®, Binab® (*Trichoderma harzianum*), Mycotal®, Vertalec® (*Verticillium lecanii*).

The products were aggregated to the 3 main groups of microbiological pesticides *B. thuringiensis*, *T. harzianum*, and *V. lecanii*.

For each examination, each person was classified as exposed if the particular product was used according to the greenhouse owner within the last year. Additionally, persons who reported that they had either dispensed or sprayed the particular product were classified, whereas if they had only irrigated the plants with a solution of the products less than 6 times a month they were assigned an intermediary exposure, while if they had mixed or sprayed the products more than once a month, or had irrigated more than 5 times a month they were assigned as highly exposed for the particular year.

The time of exposure was calculated from information from the greenhouse owners during he previous survey in 1996, from the year of introduction of the particular product in the greenhouse, or alternatively the time of employment in the greenhouse.

Statistical analysis. The IgEs to the 3 types of MBCA were transformed to binary values with a cut-off of 0.025 OD [5]. Prevalence rate ratios (PRR) were tested for each run (year) against exposure the previous year using Poisson regression analysis supplied with a cross-sectional time series analysis (xtpoisson) to see change over time in the PRR.

Prevalence rates for the baseline of the total population and the last sample of those participating in either run 2 or run 3 of each main symptom were calculated. Each symptom was tested against the exposure estimate for each of the 3 different types of MBCAs separately in a standard logistic regression analysis. A control analysis including all 3 types in the same model did not change the estimates. In the model, gender, smoking habits (smoked within the last year), and atopic status (positive reaction to 1 or more standard prick tests) were included as standard. The number of missing outcome values amounted to 0-3 observations per variable. These were list-wise deleted from the regression analyses.

The incidence of new symptoms was detected by an occurrence of the symptoms in run1-3, provided the symptom was not reported in the previous run. The time of occurrence was set to the date of the examination. However, for 6 main symptoms (cough, cough with phlegm, chest tightness, wheeze, rhinitis, and asthma) in the case that an incident symptom was registered at the examination, the time of occurrence was set to the date of the first occurrence in the quarterly mailed questionnaire. The incidence of each symptom was tested in a Cox regression analysis, including the exposure variables (3 levels, collapsing the 2 levels of applying the MBCA), gender, smoking habit, and atopy status (1 or more positive prick tests).

For asthma, an epidemiological score was calculated, slightly modified from that based on the European Community Respiratory Health Survey, composed of 7 of the original 8 symptoms (asthma attack within the last year was not included in the actual study) [15]. This score, yielding a much-skewed distribution, was tested using a cross-sectional time series analysis based on a negative binomial distribution (xtnbreg).

The lung function variables FEV_1, FVC, and FEV_1/FVC of the first and the last measurement were analysed by linear regression, including gender, age, height, smoking habits, status of atopy, and exposure within the last year. The decrease in the values per year from the first to the last measurement was calculated for each of the variables and analysed in a linear regression including the above-mentioned variables.

Bronchial reactivity was presented as the slope calculated as $\log((FEV_{1,init} - FEV_{1,last})/ FEV_{1,init} /\text{cumulated dose})+1)$ for each observation, and analysed using a cross-sectional time series analysis based on linear regression. The values were log transformed in order to get a normal distribution.

Estimates and their 95% confidence intervals were presented. All calculations were made using the statistical package STATA version 10.0 (STATA Corporation, College Station, TX, USA).

RESULTS

Exposure. The frequencies of exposure to the 4 different groups of MBCAs are shown in Figure 2. As shown, only about 10% performed work in greenhouses where no MBCA was used, while the majority persons were exposed to more than one type of agent. The different types occurred independently, with the exception f *T. harzianum* which was observed seen in the group also exposed to *B. thuringiensis*. The number of persons applying at least 1 type was 14%, while 2% applied 2 types in the same year; the application of 3 different types was only recorded once.

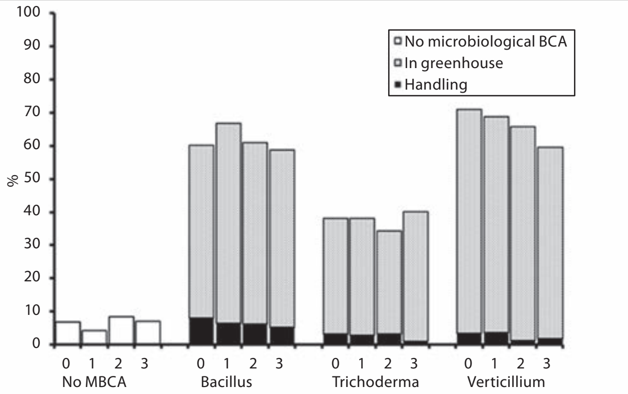

Figure 2. Distribution of exposure to the four different types of MBCA in the four 1-year periods preceding each annual examination (run 0 – run 3). The block of columns on the left shows the percentage of persons without any MBCA in the greenhouse

IgE against MBCA. The number of samples with positive specific IgEs is shown in Table 2.

B. thuringiensis. 53% of the samples had IgE values above the detection limit. A significant, although moderately increased sensitization was seen in the exposed group. Further analyses did not show a direct dose response as the rate was not higher in those who had actually handled

Table 2. Prevalence rate of positive IgEs against the three types of MBCA according to exposure within the last year. A positive IgE was defined as exceeding 0.025 OD values. Below, the crude prevalence rate ratios for sensitization are shown with 95% confidence intervals. **Bold** values are significantly different from unity (p<0.05)

		Run0	Run1	Run2	Run3
Bacillus thuringiensis	Not exposed /Exposed	52%/62%	43%/56%	43%/58%	39%/55%
	Prevalence Rate Ratio	**1.20 (1.01-1.42)**	**1.30 (1.04-1.63)**	**1.36 (1.07-1.72)**	**1.43 (1.09-1.87)**
Verticillium lecanii	Not exposed /Exposed	43%/41%	31%34%	30%/38%	38%/31%
	Prevalence Rate Ratio	0.97 (0.74-1.28)	1.10 (0.77-1.58)	1.24 (0.87-1.79)	0.83 (0.59-1.16)
Trichoderma harzianum	Not exposed /Exposed	8%/9%	8%/10%	3%/3%	2%/2%
	Prevalence Rate Ratio	1.08 (0.58-2.04)	1.21 (0.66-2.2)	0.80 (0.21-3.03)	1.18 (0.24-5.76)

the products. A longitudinal analysis showed that the effect of exposure increased over time, although not reaching significance (increase in PPR pr. year 0.15 (CI95%: -0.021-0.32), p=0.09). In examining the material, this increase in PRR covers several individual changes in sensitization, both positive and negative, which is the reason why true sensitization rates cannot be estimated.

V. lecanii. In 36% of the samples IgEs were above the detection limits, but no relation to exposure was seen in any of the runs.

T. harzianum. Only 6% of the samples were above the detection limit without relation to exposure. The sensitization rates were decreased in the two last runs.

In prick test, 1 person only showed a positive reaction to *T. harzianum,* while no reaction to *V. lecanii* was seen.

Symptoms. Table 3 shows the odds ratios for the main symptoms in relation to exposure to the 3 groups of MBCA.

As seen from the Table, no significant increase in symptoms were seen in relation to any of the 3 MBCAs.

In total, 5 persons developed asthma during the observation period – 4 females and 1 male, giving an incidence rate of $4.5 * 10^{-3}$ year^{-1}. None of the cases had measurable IgEs against MBCA, while 3 were atopics, having 3-4 positive prick tests.

Table 3. Risk estimates of main symptoms in relation to exposure to *Bacillus, Trichoderma, and Verticillium* species. The first two columns show prevalence rate ratios in relation to non-exposed persons for those only working in greenhouses and for those actively applying MCBAs (logistic regression) The last two rows show incidence ratios of newly developed symptoms (Cox regression). In parenthesis 95% confidence intervals. 0 -no estimate could be made. **Bold** values are significant (p<0.05)

Bacillus thuringiensis	Initial samples (odds ratios)		Last samples (odds ratios)		Incidence rate ratio	
	Exposure in greenhouse	Application	Exposure in greenhouse	Application	Exposure in greenhouse	Application
Cough	1.03 (0.65-1.62)	0.86 (0.41-1.82)	1.58 (0.76-3.28)	1.37 (0.45-4.11)	1.23 (0.68-2.23)	1.18 (0.50-2.80)
Chest tightness	0.95 (0.57-1.59)	1.20 (0.54-2.67)	2.80 (0.94-8.32)	2.80 (0.65-12.07)	1.09 (0.56-2.10)	0.59 (0.17-2.07)
Wheeze	0.61 (0.35-1.07)	0.37 (0.13-1.06)	0.66 (0.28-1.53)	0.18 (0.02-1.50)	0.72 (0.35-1.47)	0.15 (0.02-1.13)
Asthma in the last 3 mths.	0	0	0	0	0.98 (0.18-5.25)	0
Stuffed nose	1.58 (0.73-3.42)	1.31 (0.41-4.24)	0.55 (0.19-1.60)	0	0	0
Itching eyes	1.03 (0.66-1.59)	0.84 (0.41-1.71)	0.99 (0.52-1.90)	0.81 (0.29-2.31)	0	0
Skin rash	1.01 (0.64-1.60)	1.00 (0.48-2.10)	0.85 (0.48-1.51)	0.51 (0.19-1.39)	0	0

Table 3 (Continution). Risk estimates of main symptoms in relation to exposure to *Bacillus, Trichoderma, and Verticillium* species. The first two columns show prevalence rate ratios in relation to non-exposed persons for those only working in greenhouses and for those actively applying MCBAs (logistic regression) The last two rows show incidence ratios of newly developed symptoms (Cox regression). In parenthesis 95% confidence intervals. 0 -no estimate could be made. **Bold** values are significant (p<0.05)

Tricoderma harzianum	Initial samples (odds ratios)		Last samples (odds ratios)		Incidence rate ratio	
	Exposure in greenhouse	Application	Exposure in greenhouse	Application	Exposure in greenhouse	Application
Cough	**0.56 (0.33-0.97)**	0.43 (0.10-1.97)	**0.61 (0.30-0.58)**	0.58 (0.13-2.71)	0.75 (0.42-1.36)	0.24 (0.03-1.75)
Chest tightness	0.98 (0.56-1.71)	0.34 (0.04-2.62)	**0.63 (0.26-0.50)**	0.50 (0.06-3.97)	0.57 (0.26-1.23)	0.76 (0.18-3.15)
Wheeze	1.22 (0.66-2.28)	1.71 (0.45-6.46)	0.31 (0.09-1.71)	1.71 (0.44-6.71)	0.52 (0.22-1.26)	0.81 (0.19-3.45)
Asthma in the last 3 mths.	0	0	0	0	1.77 (0.32-9.76)	0
Stuffed nose	1.58 (0.73-3.42)	1.31 (0.41-4.24)	1.59 (0.56-0.00)	0	0	0
Itching eyes	**0.58 (0.34-0.98)**	0	0	0	0	0
Skin rash	0.99 (0.60-1.65)	0.65 (0.14-3.00)	0.31 (0.12-1.85)	1.85 (0.56-6.10)	0	0
Verticillium lecanii	Initial samples (odds ratios)		Last samples (odds ratios)		Incidence rate ratio	
	Exposure in greenhouse	Application	Exposure in greenhouse	Application	Exposure in greenhouse	Application
Cough	1.47 (0.97-2.25)	1.45 (0.81-2.60)	0.94 (0.52-1.70)	0.91 (0.33-2.48)	0.76 (0.46-1.26)	0.89 (0.42-1.86)
Chest tightness	1.45 (0.90-2.33)	1.14 (0.57-2.26)	0.72 (0.35-1.48)	0.21 (0.03-1.64)	0.96 (0.51-1.78)	1.38 (0.60-3.14)
Wheeze	0.61 (0.35-1.07)	0.99 (0.48-2.03)	0.57 (0.25-1.29)	0.40 (0.09-1.90)	1.18 (0.58-2.42)	2.18 (0.90-5.28)
Asthma in the last 3 mths.	0	0	0	0	2.05 (0.39-10.76)	0
Stuffed nose	1.04 (0.54-1.98)	1.07 (0.42-2.67)	0.77 (0.26-2.32)	1.83 (0.43-7.73)	0	0
Itching eyes	1.04 (0.69-1.55)	1.01 (0.57-1.79)	0.99 (0.56-1.75)	0.49 (0.16-1.53)	0	0
Skin rash	1.00 (0.66-1.52)	1.10 (0.61-1.98)	0.95 (0.56-1.61)	1.35 (0.60-3.07)	0	0

Lung function and bronchial reactivity. The annual decline in FVC and FEV_1 in relation to exposure to the 3 types of MBCAs is shown in Table 4. The average annual decline for FVC and FEV_1 was 37.2 (95% CI.: 26.2-48.1) ml*year^{-1} and 34.1 (26.3-41.8) ml*year^{-1}, respectively. For FVC, but not for FEV_1 a significantly larger decline was seen in those exposed to *V. lecanii* during follow-up, while no other exposure related changes were seen.

Table 4. Average annual change in decline in forced vital capacity (FVC) and forced expiratory flow in 1 sec. (FEV1) in relation to exposure to *Bacillus, Trichoderma,* and *Verticillium*. Negative values mean less decline in the exposed group than in the non-exposed group. Values are corrected for gender, age, height, smoking habits, and status of atopy (0 vs. 1+ positive standard prick test). **Bold** values are significant (p<0.05)

		In greenhouse	Application
dFEV1	*B. thuringiensis*	-6.4 (-25.5 - 12.7)	-14.1 (-39.1 - 10.9)
ml/year	*T. harziani*	-2.9 (-19.5 - 13.6)	0.0 (-42.7 - 14.7)
	V. lecanii	-0.3 (-21.1 - 21.8)	-3.4 (-29.7 - 22.8)
dFVC	*B. thuringiensis*	-7.2 (-34.1 - 19.7)	-13.9 (-49.0 - 21.2)
ml/year	*T. harziani*	-1.9 (-21.3 - 25.2)	-34.1 (-74.3 - 6.1)
	V. lecanii	**-31.7 (-61.6 - -1.7)**	**-43.9 (-80.5 - -7.3)**

Only 36 (2.3%) of the total of 1,558 measurement showed a positive PD_{20} with a decline in FEV_1 of more than 20%, and of these only 4 PD_{20} values were below 0.1 mg. In all, 184 (11.8%) showed a decline of more than 10%. Positive bronchial hyper-responsiveness was not related to exposure, neither measured as PD_{20} nor as log dose slope.

DISCUSSION

In the presented study, exposure to products containing strains of *Bacillus thuringiensis* in greenhouses culturing ornamental flowers gave rise to elevated IgE antibodies as a sign of sensitization to the products, while no sign of sensitization was seen in the other types of agents. On the other hand, no systematic relation to symptoms, in lung function, or in bronchial hyper-responsiveness was observed.

Sensitization to *B. thuringiensis* has been shown by Bernstein et al. [4]. We did find a relatively high prevalence of sensitization and some indication of a dose-related increase over time. However, we found neither exposure nor sensitization related to any measurable symptoms or deterioration in lung function. It has been debated whether *B. thuringiensis* can colonize the gut and further give rise to clinical infections as it is closely phylogenetic related to *B. cereus* where selected strains give rise to serious gastrointestinal infection [3, 16]. In the presented study, specific antibodies against the insecticidal CRY- protein were not tested. in mice Tracheal instillation of either *B. thruingiensis* var *israliensis* or *Kurstakii* revealed a dose-related acute as well as sub-chronic effects measured as patchy interstial lymphocytosis, even 70 days after cessation of exposure [17].

Antibodies against *V. lecanii* have been detected in a single study, but not related to any symptoms [6]. We found a wide range of levels of IgE against this fungus, but without relation to exposure. More than 60% of the population were employed in greenhouses where this fungus was used, as well as contamination from other sites; alternatively, cross-reactivity to other agents may be the cause of this finding.

The frequency of exposure to *T. harzianum* was lower than that of the other types, and no sign was found in the presented study of sensitization, neither in prick test, IgE, or any symptoms. Only a single study has dealt with *T. harzianum* spp. finding a sensitization rate of 27% measured by skin prick test in patients referred for allergic diseases [9]. This remarkable difference may be caused by differences in climate and thereby exposure. In our study, there was a more than 20% prevalence of atopy based on positive standard prick tests. Hence, even with a low exposure, some sensitization should be expected, and exposure measurements performed in a study carried out in parallel with the presented study showed considerable concentrations of *T. harzianum* during handling of the concentrated powder and mixing [18]. In contradiction to the above-mentioned article, a limited placebo-controlled exposure study including 8 persons with building related symptoms in water damaged schools infested with *T. harzenianum,* did not show any measurable effect of exposure on subjective rating, lung function, or various inflammatory markers [19].

The main limitation in the presented study was that the individual assessment of exposure was been crude, based only on information from the individuals and from the company about the use, but not on direct measurements of exposure. The group of persons not exposed to any of the MBCAs was also small. On the other hand, the 3 different species investigated are of very different types, with no overlapping antigenicity, which is the reason they can be treated as independent exposures with sufficient size of groups and contrast in exposure to reveal a possible health effect.

A remarkably low frequency of bronchial hyper responsiveness was seen in the presented cohort. This may imply a healthy worker selection, as those participating in the follow-up were mostly employed throughout the whole year. Therefore, persons with respiratory symptoms may have found other trades. However, the rate of atopy in the cohort did not differ from the general population, and the frequency of symptoms was not extraordinarily low, which speaks against a large healthy worker effect.

The use of MBCAs has been an established technology in the studied greenhouses for many years before the presented investigation, and only limited change in the use was seen during the study period. Therefore, most of the participants had been exposed for several years before our investigation and we had no possibility to investigate the increase in sensitization and development of health effects immediately following the first exposures and thereby estimate true incidence rates of these phenomena. Some increase in sensitization to *B. thuringiensis* was observed during the period, even at an already high sensitization rate. This may indicate that exposure still gives rise to a biological reaction even at this stage, although not affecting symptoms or physiological measurements.

In conclusion, an increased sensitization rate against species of *B. thuringiensis* was found, but not to the other microbiological control agents. No exposure-related health effect could be detected. The present cohort, to some extent, may have become adapted to possible transient health effect, but probably not to an extent that could hide a possible inflammatory effect in the airways.

Ethics approval

The study was approved by the Regional Scientific Ethical Committee of Fyns and Vejle counties, Denmark (Jr. No. 97/46). Data handling was approved by the Danish National Data Board.

Acknowledgements

The study was supported by the Pesticide Research Fund ofthe Danish Environmental Protection Agency and the Danish Strategic Environmental Research Programme.

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