

## BRIEF REPORT

# Entomopathogenic Fungus *Cordyceps militaris*: Distribution in South Lithuania, 'in vitro' Cultivation and Pathogenicity Tests

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## Abstract

*Cordyceps militaris* (L.) Link. is the type species of *Cordyceps* (Hypocreales, Ascomycetes), which parasitizes larva or pupa of lepidopteran insects. The aim of the present study was to examine: distribution of *C. militaris* in pine stands of South Lithuania, pathogenicity of the fungus, and optimal conditions for its cultivation *in vitro*. The efficiency of *C. militaris* preparations against the pine moth (*Dendrolimus pini* L.) was tested by artificial infection of hibernating insect larvae in forest litter under natural conditions. During 2010–2011, in pine stands of South Lithuania, a total of 4 % (on areas without pest outbreak) and 59 % (in areas with pest outbreak) of all hibernating insects were infected by *C. militaris*; meanwhile 99 % of insects damaged by the fungus were *D. pini*. A massive formation of stromata with fruitbodies of the fungus started at the third year (2011) of outbreak comprising 21% of all larvae infected by the fungus. Stromata with fruitbodies on *D. pini* larvae were produced after 70 – 80 days of growth under favourable conditions. The optimal conditions for cultivation of *C. militaris in vitro* were: temperature 18° C, daylight, inoculation with fresh pure culture fragments and cultivation on rice medium. The preparation of *C. militaris* biocontrol medium for pathogenicity tests was made after 35–40 days after the fungus inoculation on rice medium. Pure culture of the fungus was grown in Petri dish (diameter 9 cm) with medium made of 10 g of rice and 25 ml of distilled water. The mean 6–8 g of dry rice and the fungus mass (biocontrol medium) are possible to get from each Petri dish. The differences of larvae mortality between two treatment methods (spraying and dusting of forest litter with *C. militaris* preparation, when the larvae were still in crown), and the control, were statistically insignificant. It was estimated that spraying of forest litter with *C. militaris* preparation increased 3.7 times cordycepsmycosis disease of pine moth larvae, when they already were in forest litter.

**Key words:** *Cordyceps militaris*, impact, infestation, *Dendrolimus pini*, pests, pine stands

## Introduction

*Cordyceps militaris* (L.) Link. is the type species of *Cordyceps* (Hypocreales: Ascomycetes), which parasitizes larvae or pupae of lepidopteran insects, and has a worldwide distribution (Ильинский and Тропин 1965, Евлахова 1974, Glovacka-Pilot 1974, Kryukov et al. 2011, Shrestha et al. 2012). All *Cordyceps* species are endoparasitoid (entomopathogenic fungi), mainly on insects and other arthropods; a few species are parasitic on other fungi (Shrestha et al. 2004). Entomopathogenic fungi common in forest soils (Brownbridge et al. 1993) and have been isolated from the interface between leaf litter and the organic layer of soil (Hajek et al. 2000).

*Cordyceps militaris* infects and its mycelium subsequently colonizes body of an insect: spores of the fungus germinate and produce a germ tube that penetrates the cuticle, allowing the mycelia to grow inside the host body, as a rule with the lethal outcome (Tanada and Kaya 1993, Inglis et al. 2001).

In the southern pine forests of Lithuania, *C. militaris* for the first time was detected in 1997; however, the possibility of using this fungus in forest protection so far has not been investigated. However, it was noted that pine moth *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae; abbreviation *D. pini*) larvae are sensitive to infection by *C. militaris* (Gedminas 2000, Gedminas et al. 2000, 2011).

The pine moth *D. pini* L. is one of the most serious defoliators of pine in Lithuania. The massive outbreaks of *D. pini* in South Lithuania were first recorded in 1994-1996, and e.g. about 28,000 hectares of pine stands were damaged in 1995. The next outbreak of *D. pini* occurred in 2009, when about 7,000 hectares of pine stands were damaged. In Lithuania, *D. pini* hibernates in the larval stage (Gedminas and Žiogas 2000), while in other geographic areas, this insect might also hibernate in pupal stage (Kolk and Starzyk 1996). Females deposit to 400 eggs in groups of 20 to 150 on needles, branches and bark, and up to 50-60 needles could be grazed per caterpillar per day. Therefore, number of hibernating *D. pini* individuals in a given forest area could provide a base for prognosis how many needles will be lost next spring. For example, if the pine moth mean number reaches about 40 hibernating individuals per 1 m<sup>2</sup> of forest litter, the pine will lose 100% of needles next spring. The pine, which has lost 100% of needles, stops to grow for 6 years. After such damages the economic loss in forestry are significant (Gedminas and Žiogas 2000).

Presently, chemical and biological insecticides are the most effective method for *D. pini* control (USDA 2012, Moeller and Engelman 2008, Woreta and Malinowski 1998). Pesticides used to control *Dendrolimus* moths are primarily pyrethroids, insect growth regulators or biopesticides, and are applied as Ultra Low Volume (ULV) formulations to increase their coverage and efficacy (Sierpinska 1998, Sierpinska and Sierpinski 1995). Among bacteria, *Bacillus thuringiensis* preparations are the most widely used to control *D. pini* (Moeller and Engelman 2008, Sierpinska 1998). However, increasing resistance of pine moth to pesticides and concern regarding negative effects of the chemical pesticides on the environment had launched the search for alternative control methods, including landscape modification, host-targeted measures, and the use of *D. pini* parasitoids, predators, and pathogens. Of the latter, the biological control methods using entomopathogenic fungi seem to be the most promising because these fungi might be effective and are environmentally friendly (Malinowski 2009, Sierpinska 1998). Therefore, application of entomopathogenic fungi to soil surfaces (on forest litter) might be a suitable method for controlling pine moth populations. Moreover, interest in *in vitro* study of entomopathogenic fungus *C. militaris* has increased due to their valuable bioactive compounds and biocontrol effects (Sierpinska 1998, Gedminas 2000).

De Bary was the first who studied *in vitro* the growth of *C. militaris* by inoculating the larvae of *Sphinx euphorbiae* with ascospores and obtained stroma with immature perithecia (De Bary 1887). Subse-

quently, a number of culture techniques for fungus *C. militaris* have been tested, for example, storage/stock culture, pre-culture, popular/indigenous culture (spawn culture, husked rice culture and saw dust culture) and, special/laboratory culture (shaking culture, submerged culture, surface liquid culture and continuous/repeated batch culture) (Das et al. 2010). In addition to insect and other mycological media, producing *in vitro* stromata of *C. militaris* was reported by Kobayasi (1941), in rice medium containing 10 g of rice and 25 ml of distilled water in 100 ml Erlenmeyer flask. Furthermore, Sung (1996) developed a simple liquid inoculum method for large scale fruiting of *C. militaris* in rice medium. Effects of different additives, such as organic nitrogen sources, including yeast extract, on fruiting of *C. militaris* in rice medium were studied in 100 g of brown rice in 160 ml of water as the basal medium for fruiting (Liang 1990, Pen 1995). Effects of environmental factors on mycelial growth and fruiting of *C. militaris* were also studied (Choi et al. 1999, Sung and Shrestha 2002). Many authors have documented that the lower temperature of 18-20°C is optimum for *in vitro* fruiting of *C. militaris*.

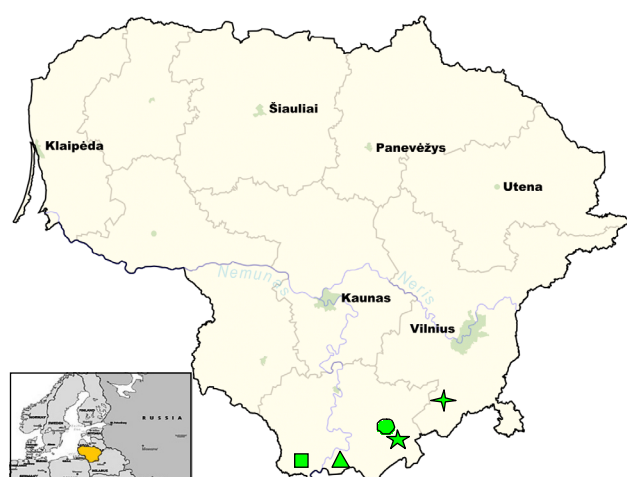
The aim of presented study was to examine the distribution of *C. militaris* in pine stands of South Lithuania; biological properties, optimal conditions for *C. militaris* growing *in vitro* and pathogenicity of the fungus to insects, focusing on *D. pini*. Data derived from such surveys have a potential to develop future biological control strategies that more effectively use naturally abundant entomopathogenic fungi to reduce pest insect populations.

## Materials and Methods

### Distribution of *C. militaris*

In order to detect *C. militaris* distribution, the infectivity of hibernating defoliators and other pests was estimated in different regions of Southern Lithuania in 2010-2011. Five research plots were selected in each of three forest enterprises: Valkininkai, Druskininkai and Veisiejai. Additionally 10 research plots were selected in Varėna Forest Enterprise in *D. pini* outbreak area. Of those, five research plots were set as the control, where treatment against pine moth has not been done. The rest five plots was as sprayed and pines were treated with biological insecticide Foray 76B (*Bacillus thuringiensis kurstaki* (Valent BioSciences Corporation, USA)). The total number of the research plots was 25 (Figure 1).

Research plots represented by middle-aged (60-70 years old) stands of Scots pine (*Pinus sylvestris* L.) stands growing in oligotrophic soils of normal moisture. Twenty pines were randomly chosen in each



Research plot No.	Kapčiamiestis	Druskininkai	Varėna (Control)	Varėna (Treated)	Valkininkai
1	53° 59' 4.82", 23° 38' 50.59"	53° 58' 16.92", 24° 3' 24.71"	54° 10' 12.39", 24° 30' 21.5"	54° 10' 33.51", 24° 34' 15.78"	54° 21' 2.22", 24° 57' 54.12"
2	53° 58' 14.87", 23° 38' 5.66"	53° 58' 13.2", 24° 4' 8.14"	54° 10' 40.38", 24° 30' 41.46"	54° 9' 28.54", 24° 34' 26.04"	54° 21' 10.5", 24° 58' 23.4"
3	53° 58' 1.53", 23° 37' 49.4"	54° 2' 30.18", 24° 4' 14.57"	54° 11' 13.66", 24° 34' 6.26"	54° 9' 12.31", 24° 34' 29.17"	54° 21' 14.13", 24° 58' 30.01"
4	53° 57' 45.46", 23° 37' 29"	54° 1' 36.56", 24° 12' 52.25"	54° 11' 36.49", 24° 34' 1.83"	54° 8' 2.26", 24° 34' 36.94"	54° 21' 40.67", 24° 59' 37.98"
5	53° 57' 34.42", 23° 37' 31.35"	54° 0' 15.26", 24° 14' 1.09"	54° 10' 55.07", 24° 34' 8.63"	54° 7' 22.5", 24° 31' 45.54"	54° 21' 53.28", 25° 0' 9.94"

**Figure 1.** Map of Lithuania (position shown by shading on the north European map in lower left corner) showing study territories. The number of plots and geographic coordinates of each plot are presented below

plot and two 50 × 100 cm sample sites were set near the base of every tree trunk. In total 1,000 sample sites were evaluated each year in October comprising 500 m<sup>2</sup> of forest litter. On every plot, layer of moss was removed and all healthy and fungus-infected hibernating larvae and pupae of pine defoliators were counted and collected. Collected samples were placed into 12 × 12 × 6 cm plastic boxes with lids containing moss (*Pleurozium schreberi* (Brid.) Mitt., in which *C. militaris* occurs under natural conditions), and kept in the refrigerator at +5 °C. The moss was collected at the same plots simultaneously while collecting the larvae. Collected insects were checked every 10 days, and for each sample a total of 8 checks have been done.

#### *Cultivation C. militaris in vitro*

*C. militaris* was grown on infected pine moth larvae under laboratory conditions. Fifty dead larvae were collected in the summer of 2010 (after infection in autumn and hibernation). Another fifty, but living, larvae have been collected in the autumn of 2010 and were freshly infected by the fungus. Each infected larva was placed in a closed transparent container filled with moss, which regularly were irrigated with distilled water. Larvae were kept under natural lighting conditions; air humidity was 80-95% and air temperature 15-18 °C. During the investigation, the growth phase,

stage of development and morphological changes in the fungus *C. militaris* were recorded. The amount (number) and size (length) of stromata were measured on each larva.

In addition, the fungus *C. militaris* was grown on the artificial nutrient medium. For fungal medium preparation we used the modified methodology of Kobayasi (1941). Artificial medium was prepared using 10 g of rice and 25 ml of distilled water per Petri dish. We used glass Petri dishes (9 cm diameter × 1.8 cm height). Dishes with rice and water were placed in the drying oven at the temperature of 120 °C for 60-100 min. Pure *C. militaris* fungal cultures were isolated from dried and non-dried hyphae fragments, stromata fragments, ascospores, mycelium parts and internal organs of an infected larvae. In order to determine the optimal conditions for *C. militaris* growth in artificial medium, the cultures were grown in the dark and under different light regimes at the temperature of 5 °C, 10 °C, 16 °C and 18 °C in three replicas (15 Petri dishes in each replica). Fungal growth, its morphological features, medium humidity and contamination of the nutrient media by other fungi were recorded every week until the fungus completely covered the surface of nutrient media in 9 cm diameter Petri dish. Fungal powder for pathogenicity tests was made triturating the dried fungus mycelium (conidia and hyphal bodies) together with the rice medium, where the fungus was grown. One gram of the fungus powder composed of  $4.13 \times 10^9$  conidia and additives (hyphal bodies and particles of rice medium).

#### *Pathogenicity tests with C. militaris*

The experiment was performed in order to detect *C. militaris* pathogenicity on hibernating insects in the forest litter in Varėna forest enterprise) in October 2010. Plastic containers were prepared, 16 cm width, 40 cm length and 14 cm depth. The layer of soil covered with a layer of moss was added in the each container (litter simulation). In total 30 containers were prepared. The mosses in containers were treated with lab-cultured fungal preparations in two different ways: i) dusting fungal powder (norm 4.7 g/1 m<sup>2</sup>) (10 containers) and, ii) spraying the *C. militaris* fungal suspension (norm 24 ml/1 m<sup>2</sup>) (10 containers). Fungal suspension was made from 1 g of fungal powder mixed with 1 l of distilled water. Ten remaining containers were controls, without fungal infection. Ten symptomless pine moth larvae (3<sup>th</sup> – 6<sup>th</sup> instars), varying in different development stages, were placed in the each container. In total 300 larvae were placed in hibernating containers, which were installed in the forest litter within the moss surface in October 2010 and left under natural conditions until the next autumn (2011). The

number of larvae infected with *C. militaris* was determined in October 2011. Larvae classed as infected were covered either with *C. militaris* mycelium or with fungal stroma.

During October 2010, another experiment was conducted in the pine stands subjected to outbreak of pine moth in 25 research plots. Of those, 15 were of 9 m<sup>2</sup> in size and 10 were of 100 m<sup>2</sup>. The forest litter in the plots of 9 m<sup>2</sup> was treated with the fungal preparation in two ways: dusting with fungal powder (2.2 g/m<sup>2</sup>); spraying with water suspension of the fungus, which concentration was 1 g/l (24 ml/m<sup>2</sup>). This experiment was done, when the larvae were still in crowns (in October 2010). The forest litter in the research plots of 100 m<sup>2</sup> was treated with fungal preparation by spraying, when the larvae were hibernating in the forest litter (November 2010) in five replications. Control plots were sprayed with distilled water. The observations of larvae contamination were made one year later (October 2011) in each research plot. Accounting plots in size of 1 m<sup>2</sup> were set in the 9 m<sup>2</sup> and 100 m<sup>2</sup> research plots, respectively, under one and five pine trees. The data were treated statistically using basic statistical methods (Čekanavičius and Murauskas 2000, Čekanavičius and Murauskas 2004).

## Results

### *Geographic distribution of C. militaris in South Lithuania*

During the investigation, distribution of *C. militaris* was determined under *D. pini* outbreak conditions after the aerial treatment with biopreparation Foray 76B. The larvae of *D. pini* were most abundant among defoliating hibernating insect pests in pine forest litter and comprised 81% of the all examined insects in regions of South Lithuania in 2010-2011. The other species of pine defoliators were: pupae of *Bupalus piniaria* (Lepidoptera: Geometridae) (12%), pupae of *Hyloicus pinastri* (Lepidoptera: Spingidae) (4%) and larvae of *Diprion pini* (Hymenoptera: Diprionidae) (5%). The total number of defoliating hibernating insect pests in forest litter was 570 (in 2010) and 632 individuals (in 2011), i.e. 1.1 and 1.3 individual per 1 m<sup>2</sup> of forest litter.

In 2010, the largest amount ( $4.5 \pm 1.1$  unit/m<sup>2</sup>) of defoliating insect pests hibernated in the Varėna pine stands were detected in the outbreak of *D. pini*, where the treatment with Foray 76B was not applied. The number of hibernating insects was 3.2 times less ( $0.2 \pm 0.01$  unit/m<sup>2</sup>) in forests treated with biopreparation Foray 76B in comparison with untreated ones.

The distribution of *C. militaris* was directly related to the abundance of *D. pini*. The healthy insects

**Table 1.** Morphological features of *C. militaris* from South Lithuania

	Size	Shape	Color
Stroma	10-130 x 2-8 mm	clavate	bright orange
Perithecium	600-660 x 260-330 µm	drop shaped	orange
Asci	300-510 x 3.5-5 µm	cylindrical, needle shaped	hyaline
Ascospore	317-378 x 1.25-1.60 µm	filamentous, septated	hyaline
Partspore	2.9-4.2 x 1.25-1.60 µm	ellipsoidal, cylindrical	hyaline

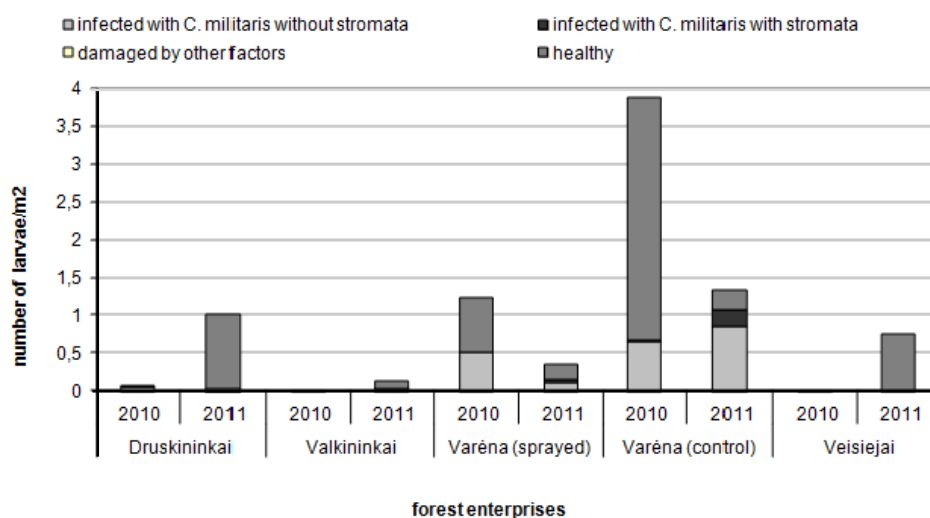
comprised 75%, infected by *C. militaris* – 24% and 1% – dead due to other factors (as entomophagous predation, mechanical damages, other fungal diseases etc.) of the all hibernating insects in forest litter in 2010. Accordingly, 78% of the insects were healthy and 21% – infected by the fungus in 2011. The main morphological features of *C. militaris* detected in pine stands of South Lithuania are presented in Table 1.

Stromata of *C. militaris* are clavate (club-shaped) and orange to yellow in colour. The perithecium of *C. militaris* (size 600-660 × 260-330 µm) covers about 0.3-2 cm of the top of stroma. Perithecia are drop shaped and located in the top layers of stroma. Tops of perithecia are near the surface and this part of stromata looks humpy. The asci and ascospores are inside the perithecia. The asci form inside and concentrate near the hole, which appears near the top of perithecia after full maturation. The asci are hyaline, long, needle shaped containers. Ascospores are thrown outside through the hole, when the perithecia of *C. militaris* split. The observed ascospores of *C. militaris* were hyaline, filiform, septated (size  $357 \pm 5.7 \times 1.43 \pm 0.04$  µm). The ascospores fall in to the separate segments after they access the surrounding. The segments of ascospores (partspores) are very small (size  $3.8 \pm 0.12 \times 1.4 \pm 0.04$  µm); therefore, they are easily transferred by wind, rain or forest animals. Each of these spores or segments of the spores germinates under the favourable environmental conditions.

The large number of hibernating defoliating insect pests damaged by *C. militaris* was found in the Varėna pine stands, where the outbreaks of *D. pini* were detected (Figure 2). The number of hibernating defoliating insect pests damaged by *C. militaris* in the Varėna pine stands, where the treatment with Foray 76B against *D. pini* was made (sprayed), was 0.51 larvae/m<sup>2</sup> (i.e. 42% of all hibernating defoliating insect pests). The number of damaged insects was 0.66 larvae/m<sup>2</sup> (16% of all hibernating defoliating insect pests) in Varėna stands, where treatment against *D. pini* was not made (control). It can be explained that outbreaks of *D. pini* in the treated plots (in 2009) formed earlier than in the untreated plots (in 2010) and fungus *C. militaris* had more time to infect *D. pini* larvae in the treated plots. In 2010, the number of hibernating defoliating insect pests damaged by *C. militaris* in Druskininkai Forest Enterprise was 0.05

larvae/m<sup>2</sup> (33%), in Valkininkai – 0.05 larvae/m<sup>2</sup> (100%) and in Veisiejai it was not found (Figure 2). After one year (2011), the number of insect pests damaged by *C. militaris* in Varėna Forest Enterprise was 1.08 lar-

vae/m<sup>2</sup>. The net of the fungus hyphae gradually starts to thicken and becomes orange in natural light cycle, while mycelium always is white (because of pigment deficit) in the dark.



**Figure 2.** The condition of pine defoliators occurred in forest litter







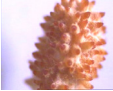
vae/m<sup>2</sup> (59%) in the control (non-treated) pine stands and 0.16 larvae/m<sup>2</sup> (46%) in treated (sprayed) stands. It has happened because the number of insects after one year was larger in the plots, where the pest control (treatment with Foray 76B) was not used. In Druskininkai the amount of damaged larvae was 0.02 larvae/m<sup>2</sup> (2%), in Valkininkai 0.03 larvae/m<sup>2</sup> (31%) and in Veisiejai insects were not damaged by *C. militaris* in 2011 (Figure 2).

The larvae of *D. pini* comprised 99 non treated % (130 larvae in autumn and 124 larvae in spring) of all insects infected by entomopathogenic fungus *C. militaris* in forest litter of South Lithuania in 2011.

#### *The in vitro* cultivation of *C. militaris*

The differences between fungal growth *in vitro* on larvae of *D. pini* collected in the summer and autumn of 2010 were insignificant. The duration of *C. militaris* development cycle is one year under environmental conditions in Lithuania. The larvae of *D. pini* infected by the fungus in late autumn (October - November) die in the running year (till frosts). According to the results of inoculation, the following sublethal phases were typical for infested larvae: a) 2-5 days after infection the larvae wimp out and lose the rigidity, but still have a passive reaction to the irritants; b) 6-10 days after infection develops mummification followed by “freeze” of the larvae, and they become involuted, crooked or prostrated (Table 2); mean duration of mummification is 7 days; c) subsequently, the net of whitish *C. militaris* mycelial layer

**Table 2.** The stages of *D. pini* infection with *C. militaris* in laboratory and in forest

	Days after inoculation		Infection stages
	In laboratory	In forest	
	0	0	Healthy larva of <i>Dendrolimus pini</i>
	2-5	2-5	Live larvae but wimp out and lose the rigidity
	5-10	5-10	Mummification of larvae
	10-30	10-300	Mycelium development on larvae; the mycelia of the fungus becomes orange
	30-65	300-335	Stroma development; the top of the fungus stroma thickens
	65-80	335-350	Perithecia formation; perithecia locate in the top layers of stroma
	80-87	350-365	Ascospores development; the asci form inside and concentrate near the top of perithecia after full maturation. Ascospores are discharged outside and fall in to the separate segments after they access the surrounding



Dead larvae of *D. pini* under the moss in forest litter showed white and clearly yellow mycelium, penetrating tissue of dead larvae. The only one or few stromata of the fungus were typically formed in late autumn (October). It was observed that 3-10 cm long larvae contain sufficient substrate size to support the development of a stroma.

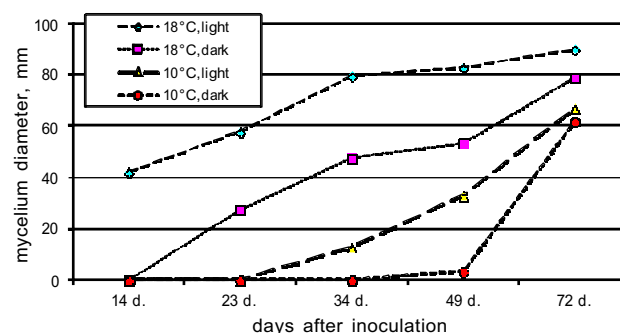
The mycelia of the fungus became dark and orange after 25-30 days from the infestation. The orange outgrowths, aggregation of the fungus hyphae (0.5 cm long), developed one week later. The stromata of the fungus developed only from the largest outgrowths of the mycelium. After additional 10 days, the stromata became 1 cm long. Later, the fast growing of fungal stromata proceeds. The classical form of a stroma is the shape of mace or distaff (thickened to the end). The top of the fungal stroma is spiky in the beginning of the development) and this feature may help to penetrate through the moss. The top of the fungal stroma thickens during 30-35 days from the beginning of stroma development. At the same time, the fruit bodies, perithecia of the fungus, are developing on the top of the stroma. It was clarified that *C. militaris* from the beginning of the experiment to the maturation of fruit bodies developed during 70-80 days (under natural lighting conditions, air humidity of 80-95% and temperature of 16-18 °C) on the larvae of *D. pini* (Table 1).

The stroma of *C. militaris* grows commonly from that part of the larval body, which lies on the soil. The size and amount of fungal stromata depend on the size of larvae. Only one and big stroma grows up from the *D. pini* larvae of the 5<sup>th</sup> to 6<sup>th</sup> instars. The fruit body of *C. militaris* of 13 cm in length was found during the research in the Varėna pine forests. Therefore, the stromata of *C. militaris* are able to develop not in all infected larvae. The fungus can infect the larvae and stay at mycelial stage until the next autumn. During this period the fungus penetrates the tissue of dead larvae. Stromata begin to grow then the amount of organic mass is sufficient for the fungus development. The stroma of *C. militaris* starts to grow immediately (in laboratory) from the infected larvae, which were collected in spring. The stromata from the larvae left under natural conditions (forest litter) grow up only in autumn. It was determined that the stroma of *C. militaris* can be grown as from freshly infected *D. pini* larvae as from formerly (10 months ago) infected larvae.

During our observations, it was detected that the inoculations depends from larval stage during the period of hibernation. In Lithuania, *D. pini* usually hibernates at the larval stage, and other lepidopteran species at the stage of pupae. According to our results, the pupae of other lepidopteran species were more rarely infected by *Cordyceps* mycosis. Therefore,

the larvae of several Lepidoptera species go to hibernate before the fruit bodies with ascospores of *C. militaris* mature.

The best growing results of *C. militaris* were obtained, when the fragments of fungal stroma were cultured on the rice media. The tests were conducted at the temperature of 5, 10 and 18 °C, respectively. The growth of *C. militaris* was very slow at air temperature of 5 °C. It was established that low air temperature (10 °C) did not finally stopped the growth of *C. militaris* mycelium but almost eliminated (in 75%) non-target fungi and bacteria. Mycelia growth at a temperature of 10 °C began two weeks later than at a temperature of 18 °C (Figure 3). During 34 days after inoculation, the diameter of *C. militaris* colony was 1.3 cm (at the temperature of 10°C under lighting). During 72 days, the mycelia diameter was 6.7 cm. The higher air temperature (18 °C) and natural lighting conditions were more effective for *C. militaris* mycelia growth. Two weeks after inoculation, the mean diameter of mycelium was 4.2 cm. The most Petri dishes were 100% covered by the fungus mycelium (mean diameter 9 cm) 72 days after inoculation. Darkness was a limiting factor for mycelium growth. In the beginning, the mycelium of the fungus is white and within 10 days



**Figure 3.** The growth of *C. militaris* mycelium under different conditions (air temperature, light)

after culturing it became yellow (under lighting conditions). After the following 24 days, the mycelium became orange. It has been well documented by many authors (Sung 1996, Choi et al. 1999, Sung et al. 1999), that the lower temperature of 18-22 °C is optimum for *in vitro* fruiting of *C. militaris*. In our experiments, the higher air temperature than 18 °C was followed by media contamination with bacteria.

The tested conditions for *in vitro* growing *C. militaris* are as follow: artificial rice media, 18 °C temperature and natural light were favourable. It is possible to get pure culture of *C. militaris* during 35-40 days under laboratory conditions, which are favourable for development of the fungus. The mean 6-8 g of

dry rice and the fungus mass are possible to get from the each Petri dish (on 10 g rice and 25 ml distilled water). During the research, some antagonistic features of *C. militaris* to some mould fungi were observed. The parasitic fungus *Melanospora parasitica* Tul. (class: Ascomycetes, family: Melanosporaceae) was identified on *C. militaris*. *C. militaris* cultures contaminated with *M. parasitica* were directly destroyed.

#### The pathogenicity of *C. militaris*

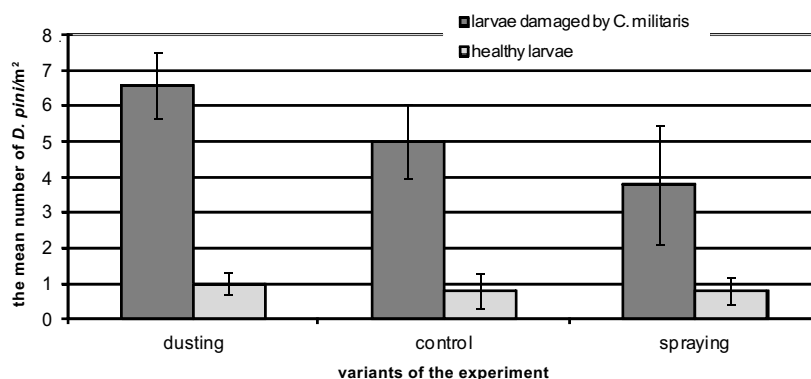
The preparation of *C. militaris* (cultured in the laboratory) used in field investigation in October of 2010. Pathogenicity of the fungus was assessed one year later. The results were very similar and independent of the type of *C. militaris* application (dusting or spraying) on 9 m<sup>2</sup> forest litter plots, when the larvae were still in crown. The mean mortality of larvae of *D. pini* (from the disease) was  $6.6 \pm 0.92$  larvae/m<sup>2</sup> after dusting and  $3.8 \pm 1.68$  larvae/m<sup>2</sup> after spraying (Figure 4). However, the differences between these two treatment methods was statistically insignificant ( $t = 1.45$ ,  $p = 0.2$ ). The differences between *D. pini* larvae mortality in the control (spraying with distilled water) and dusting plots are statistically insignificant ( $t = 1.14$ ,  $p = 0.3$ ). Before the experiments the mean number of *D. pini* larvae hibernating in the forest litter was  $5.8 \pm 0.76$  larvae/m<sup>2</sup>.

Spraying with *C. militaris* preparation was more effective (3 times) in 100 m<sup>2</sup> plots, when the larvae was already in the forest litter than spraying when larvae were still in the crown of pines. The main reason of this could be the faster contact of the fungus and larvae in the forest litter. The longer absence of contact between the fungus and larva significantly reduces the potential of fungal germination.

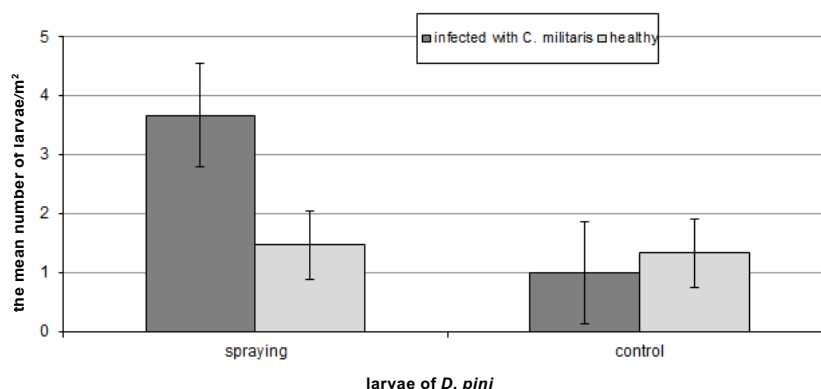
It was estimated that the number of infected larvae in sprayed research plots, when the larvae was already in forest litter, ( $3.6 \pm 1.05$  larvae/m<sup>2</sup>), than in the control plots ( $1.0 \pm 0.57$  larvae/m<sup>2</sup>) (Figure 5). This difference is statistically significant ( $t = 2.84$ ,  $p = 0.01$ ).

The number of infected *D. pini* larvae was similar (insignificant differences) in hibernating containers after spraying with *C. militaris* preparation and on control sites. During the experiment, 21% of the all infected larvae were found with stromata of *C. militaris*. The number and size of the stromata differed very much. When many stromata produced on a larva, their sizes tend to become shorter while they were produced in small number, they tend to become longer. It is limited amount of nutrients necessary for fungus *C. militaris* stroma formation in the body of pine moth larva. Therefore, the one large stroma (with perithecium) or several smaller stromata (without perithecium) were detected per one larva.

**Figure 4.** The mean number of *D. pini* larvae infected with by *C. millitaris* (preparation was used before insects hibernation, when the larvae were still in crowns)



**Figure 5.** The mean number of *D. pini* larvae infected with *C. millitaris* (preparation was used in time of insects hibernation, when the larvae were in the forest litter)



## Discussion and Conclusions

It has been ascertained that the distribution of entomopathogen *C. militaris* in the forest litter depends on its insect hosts as the pine moth abundance. Results have shown that 99% of the insects damaged by the fungus were larvae of *D. pini* in 2010-2011. It has been estimated that infected by *C. militaris* pine moth larvae were 3.6 times more in the insect outbreak than on the control site. It shows the importance of the fungus in the self-regulatory processes of forest ecosystems. According to the classic needle chewing instars as developmental stages, it would be called climax phase (III) or reduction in pest abundance (IV) (Ильинский and Тропина 1965). In the previous research (1996-1999), it was noticed that 67% of larvae were infected by *C. militaris* at the climax stage of pine moth outbreak (Gedminas 2000, Gedminas et al. 2000).

It can be argued that the *C. militaris* is pathogenic to the pine moth larvae and slightly pathogenic to other pine defoliators hibernating in the litter of pine forest. We did not detect any other insects (except the pine moth larvae) infected with entomopathogenic fungi; although the literature sources indicate that *C. militaris* infects many species of moth larvae and pupae (Sato and Shimazu 2002). The low infection of other insects by *C. militaris* may be related with exchanges of phenological and development stages of pine defoliators. *Panolis flammea* and *Hyloicus pinastri* go to hibernate in the forest litter before the fruitbodies and ascospores of *C. militaris* appeared. This strain of *C. militaris* is specialized entomopathogen for *D. pini* because the fruitbodies are formed exactly at the time, when the pine moth larvae go to forest litter for hibernation (from the beginning of October till the frost). The infection depends on the stage of insect development during hibernation. In addition, all of our monitored hibernating insects, except pine moth, hibernated at pupae stage and pupae are more resistant to *Cordyceps* mycosis disease than larvae (it may be because larva drops its upper layer covered with ascospores of *C. militaris* during the transformation to pupa) (Gedminas et al. 2011). It was found in the other literature sources that fungus *C. militaris* is found on the pupae more often than on larvae (Mains 1958). We did not confirm this in our research. Other authors indicate that *C. militaris* was detected on the *Tipula paludosa* Meig. larvae (Muller-Kögler 1965). One more reason for infection by *C. militaris* depends on larvae hair. Haired larvae of *Dendrolimus pini* are covered with more spores of entomopathogenic fungus than hairless larvae of other insects.

It was detected that optimal conditions for *C. militaris* mycelium growing in the laboratory are as fol-

low: air temperature of 18 °C, natural daylighting, inoculation made from the pure culture of mycelium fragments and cultivation on rice medium. *C. militaris* can be multiplied *in vitro* after 35-40 days under favourable conditions for the fungus growing. However, in such conditions only conidia of the fungus were produced. In the studies of a Japanese scientist (Kobayasi 1941), the perithecia of *C. militaris* were produced on the artificial rice media after four and half month after inoculation. The rice medium was prepared in a different way than in our experiment. The medium was autoclaved twice and inoculation was made using ascospores. In our case, *C. militaris* stroma with fruitbodies was produced 3 months after pine moth larvae inoculation with the fungus ascospores. It is supposed that a temperature around 20 °C is suitable for mature stroma production. Stromata production of *C. militaris* has been recorded at 20 and 25 °C (Harada et al. 1995). Basith and Madelin (1968) reported that 18-22 °C was suitable for stroma production, and they also recorded stromata initiation at 23 °C. Other authors (Choi et al. 1999, Sung and Shrestha 2002) found that optimal temperature for fruiting of *C. militaris in vitro* is 18-20 °C. The stroma production of *C. militaris* was examined in Japan by injecting a suspension of its hyphal bodies into three species of lepidopteran pupae (*Mamestra brassicae*, *Spodoptera litura*, *Bombyx mori*) and a species of coleopteran pupae (*Tenebrio molitor*). *C. militaris* killed the pupae of all four insects and produced mature stromata from them in the temperature interval between 20 and 25 °C, showing a shorter maturation period at 25 °C (Sato and Shimazu 2002).

During the laboratory test it was evaluated, that on 10 g of rice medium with 25 ml of distilled water there were obtained 6-8 g of dried mass of *C. militaris* (mixture of rice and fungus).

Prepared fungal powder was used for inoculating the media and other insects at different stages of their development. The fungus preparation was active and effective because many pathogenicity tests were positive (Gedminas et al. 2011). Therefore, it can be used for forest protection as a preventive tool against pests hibernating in the litter. The specificity of fungal preparation to damage only larvae of *D. pini* restricts its use but protects non-target forest insects at once.

A massive formation of stromata with fruitbodies of the fungus started in the third year (2011) of outbreak formation and comprised 21% of all larvae infected by the fungus. *C. militaris* has one year development cycle in Lithuania. The pine moth larvae die in the same year before the frost after infection in October-November. A short fatal period (5-7 days) indicates that this *C. militaris* is an aggressive patho-



gen. *C. militaris* stroma ranging from 0.8 cm to 10 cm in length in general (Евлахова 1974, Ильинский and Тропин 1965) but we found stroma 13 cm in length (Varėna forest). According to other authors (Mains 1958, Евлахова 1974, Hywel-Jones 1994, Sung and Spatafora 2004) the size of *C. militaris* varies as follows: stromata 15-30 × 0.9-4 mm, perithecium 500-720 × 300-480 µm, asci 300-510 × 3.5-5 µm, partspore 2-4.4 × 1-1.5 µm.

After formation of several stromata, they usually are smaller and, in this case, cannot always penetrate the moss on the surface. The stromata of *C. militaris* do not form in infected larvae anytime. In this case, the fungus can remain at mycelium stage. However, at this stage, the fungus spreads and forms more mycelium (depending on the conditions in forest litter) and increases several times the possibilities of infection. This way of infection is passive and depends on larvae contact with already infected larvae during its moving for hibernating.

The results from the experiment of pathogenicity tests on pine moth larvae in hibernating containers were not reliable. The results could be more accurate if the healthy pine moth larvae are placed in containers. It is quite difficult to verify that larvae is completely healthy in nature (infection could not be seen at incipient stage). It should be noted that used containers were not tight enough, because during the counting the infected larvae were found near the container. However, the experiments with hibernating containers have shown that it is possible to create the conditions suitable for *C. militaris* cultivation and fruiting.

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