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Gypsy Moth Management with LdMNPV Baculovirus in Cork Oak Forest

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Abstract: (1) Research Highlights: Applications of a species-specific baculovirus is a promising method to control the gypsy moth and regulate its population dynamics in forest ecosystems. (2) Background and Objectives: Cork oak protection against the Lepidopteran defoliator *Lymantria dispar* requires an appropriate forest ecosystem management program, involving the application of eco-sustainable microbial products during population outbreaks. The species-specific multicapsid nucleopolyhedrovirus (LdMNPV), agent of natural epizootics in gypsy moth populations, represents an option that was investigated in a multi-year field study, involving viral applications either from the ground or by aerial treatment. (3) Materials and Methods: Efficacy trials against *L. dispar* populations were conducted in 2018 and 2019 in Sardinia, according to a randomized block design. Each year, two trials were conducted, applying a baculovirus commercial formulation with an atomizer from the ground and assessing the effects of different doses and application timing, respectively. An aerial application trial distributing LdMNPV at ultra-low volumes (2 L/ha) was also conducted in 2019 to assess the virus efficacy at a larger field scale. (4) Results: In both years, a significant increase in larval mortality was detected in plots treated with higher viral occlusion body (OB) doses and with an earlier application targeting younger larvae, in comparison with untreated controls. Due to an observed retrogradation phase of the target pest in 2019, no significant differences in larval density between areas treated from a helicopter and control were detected, but in the few weeks following application, a meaningful vitality decrease in larval samples from treated plots was observed. (5) Conclusions: Based on the results of this study, the use of LdMNPV in forest protection programs against gypsy moth can be worth consideration in multi-year integrated program strategies to modulate population dynamics.

Keywords: biocontrol; bioinsecticide; entomopathogen; microbial; ecosystem

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1. Introduction

Lymantria dispar (L.) (Lepidoptera: Erebidae), also known as gypsy moth, is a univoltine species whose larvae, hatching from overwintering eggs, cause significant damages to cork oak leaves. The combination of their feeding behavior and a high biotic potential are the cause of periodic outbreaks, determining wide forest defoliations [1]. In order to reduce such deleterious effects, the implementation of appropriate biocontainment measures is necessary. Accordingly, the application of bioinsecticides was proven to be a successful approach to counteract this pest action, ensuring limited environmental impact [2]. For this purpose, available formulations based on the entomopathogenic bacterium *Bacillus thuringiensis* exploit the highly specific mode of action of bacterial toxins selectively targeting moth larvae [3]. On the other hand, the risks of possible side-effects on non-target lepidoptera inhabiting the forest ecosystem have sometimes been reported [4]. Another group of entomopathogens is represented by baculoviruses, very specific microorganisms co-evolved with their host [5] and able to cause fatal infections to larvae after the ingestion of viral particles. The bioinsecticidal activity is associated with crystalline occlusion bodies

that, after being ingested by susceptible insects, release occlusion-derived viruses (ODVs) acting on the host midgut epithelial cells. The infection spread in the host body relies on the production of a second type of virions, namely, budded viruses (BVs) [6].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) is specifically associated with gypsy moth, being co-evolved with this species [7]. This biocontrol agent represents a natural regulator of the defoliator population, as a result of periodic viral epizootics, especially under high-density conditions [8]. Hence, the baculovirus, reproduced in the laboratory on live larval material and applied in the field, becomes a tool able to significantly affect the population dynamics and therefore be used artificially to counteract gypsy moth outbreaks [9]. While this strategy appears promising, the use of LdMNPV is relegated to specific contexts where commercial products are available (i.e., Canada, USA). Due to the high costs associated with the production of viral material and the lack of available products in the market of several global regions (i.e., Europe) where local *L. dispar* ecotypes represent a constant threat to the different tree forest species, experimental work is needed to implement the use of baculovirus in different environmental conditions.

The present study had the purpose of evaluating the efficacy of a LdMNPV formulation under use in other world areas, against European *L. dispar* in cork oak forests affected by this pest. The study was conducted through two steps in different years, involving either small-scale viral applications from the ground, and larger-scale aerial treatments.

2. Materials and Methods

2.1. Tested Formulations

A suspension concentrate formulation of LdMNPV, commercially authorized in Canada, was provided by Andermatt Biocontrol AG (Switzerland) for experimental applications from the ground or by helicopter. The concentration of the active substance was 2.8×10^{10} OB/L. Foray 76B (Sumitomo Chemical Agro Europe S.A.S), containing 20 billion international units (BIU)/L of *Bacillus thuringiensis kurstaki* (*Btk*) strain ABTS-351, was used as a reference product.

2.2. LdMNPV Applications from the Ground

Two different trials were conducted to evaluate the effects of the baculovirus: (1) time–response and (2) dose–response. Treatments and application details are summarized in Tables 1 and 2, respectively. Time–response and dose–response trials were conducted in different experimental fields in the same year.

Table 1. Treatments in the time–response trial.

Treatment	Description	Application Date		Application Rate
		2018	2019	
Untreated Check	Not treated	-	-	-
LdMNPV Early	Earlier application	9 May	4 May	2 L/ha
LdMNPV Later	Later application	16 May	11 May	2 L/ha
Foray 76B	Reference product	16 May	11 May	2 L/ha

Table 2. Treatments in the dose–response trial.

Treatment [a]	Description	Application Rate
Untreated Check	Not treated	-
LdMNPV Low	1/3 standard rate	0.66 L/ha
LdMNPV Standard	Standard rate	2 L/ha
LdMNPV High	3× standard rate	6 L/ha
Foray 76B	Reference product	2 L/ha

[a] All applications were made on one date (9 May 2018, and 11 May 2019).

The trials were conducted in 2018 and 2019 in forests in north-western Sardinia (Italy) in compliance with Good Experimental Practice (GEP) guidelines established by the European and Mediterranean Plant Protection Organization (EPPO PP 1/210(1), Efficacy evaluation of insecticides—Defoliators of forest trees). The completely randomized experimental design involved four plots (100 m^2) per each treatment. Gypsy moth larval density was recorded before treatments and during the following three weeks (7, 14, and 21 days after LdMNPV application). Assessments were based on counting the number of larvae in eight 30 cm long branches randomly sampled from each plant. Defoliation levels in plots were also evaluated after treatments.

In the time–response trial, early application was conducted one week earlier (9 May 2018, and 4 May 2019), targeting eggs and just-hatched first instar larvae, while standard applications (16 May 2018, and 11 May 2019) targeted first and second instar. In the dose–response trial, applications were made on one date (9 May 2018, and 11 May 2019). Baculovirus applications were carried out with a motorized atomizer (M3 series, Cifarelli SpA, Italy), with a volume of 10 L per plot.

2.3. Aerial Applications

Aerial applications were carried out on 11 May 2019 on a forest area located in the Centre of Sardinia (Abbasanta, Italy). Treatments were performed in ultra-low volumes (ULVs), employing a helicopter (LAMA SA 315/B) equipped with 4 electronic Micronair rotary atomizers (model AU) treating a 20 m wide lane. Treatments were performed early in the morning so that environmental conditions ranged within sub-optimal limits. During product application, a global positioning system (GPS) was employed to trace and record the helicopter route, ensuring accurate and homogeneous distribution. Untreated check plots were compared with plots (around 100 ha each) treated with LdMNPV or *Btk* (Foray 76B). Direct assessments were based on counting the number of larvae on four 30 cm long branches per each of ten plants randomly sampled in each experimental plot. In addition, samples of larvae (n = 100) were collected from each plot and maintained in the laboratory on foliage collected from the same plots after treatment, in order to compare insect survival. The experiment involved three replicates.

2.4. Data Elaboration and Statistical Analysis

Overtime differences in average larval density among treatments in application experiments from the ground were tested using repeated measures ANOVA (PROC MIXED), and means were separated by LSMEANS comparison (adjust = Tukey), using SAS software (version 9.1) [10] with the significance level set at $\alpha = 0.05$. Analysis of variance (ANOVA) followed by least significant difference (LSD) test ($p < 0.05$) was used to compare efficacy data on a specific date and defoliation levels among treatments.

For different datasets in this study, in order to verify assumptions of normality and heteroscedasticity, the Shapiro–Wilk [11] and Levene's tests [12] were performed, respectively. If necessary, data were transformed as the arcsine of the square root of the percentage.

Field treatment efficacy was evaluated in terms of larval density reduction, where percent reduction in treatment x after t days (ΔD_{xt}) was calculated as:

$$\Delta D_{xt} = \frac{D_{x0} - D_{xt}}{D_{x0}} \times 100 \quad (1)$$

where D_{x0} is the initial larval density in treatment x at sampling time 0 (i.e., before application), and D_{xt} is the larval density t days after applications in treatment x. Efficacy differences between treatments were tested separately for each sampling date (i.e., 7, 14 and 21 days after applications) using one-way ANOVA. Tukey's test at a significance level of 0.05 was used for means separation if necessary.

Aerial application trial data obtained from laboratory observations on field-collected larvae were analyzed by a mixed effects Cox proportional hazard model using survival [13], and coxme [14] packages in R software [15]. In each model, treatments were considered

as fixed factors and the larval cage (i.e., replicate) as a random effect factor. Further post hoc analysis was performed using the multcomp package in R [16], applying a Bonferroni correction for multiple testing. Moreover, larval density reduction (%) was corrected for natural mortality to take into account the effect of natural population decreases (C_{xt}) using the Schneider-Orelli formula [17]:

$$C_{xt} = \frac{\Delta D_{xt} - \overline{D}_{Ct}}{100 - \overline{D}_{Ct}} \times 100 \quad (2)$$

where ΔD_{xt} is the larval density reduction (%) in treatment x after t days, and $\overline{D}_{Ct}$ is the average larval density reduction (%) in untreated control t days after applications. After this correction, transformed data were used to evaluate the merely effect of *Btk* or LdMNPV against gypsy moth larvae as assessed in the laboratory. Student's t-test at the 0.05 level of significance was used to test for differences between different treatments 7, 14, and 21 days after application.

3. Results

3.1. LdMNPV Applications from the Ground

Comparing the different plots involved in trials, a homogeneous larval density was observed before insecticidal applications in both years for time–response (2018: $F_{3,15} = 0.31$; $p = 0.82$; 2019: $F_{3,15} = 1.33$; $p = 0.31$) and dose–response (2018: $F_{4,15} = 0.26$; $p = 0.90$; 2019: $F_{4,15} = 0.16$; $p = 0.95$) trials.

In the time–response trials conducted in 2018, no significant changes in larval density were observed one week after the application of LdMNPV in the "LdMNPV early" experimental thesis. On the other hand, a significant larval density reduction was found during the following two weeks in the same plots, in comparison with the untreated check ($F_{9,63} = 15.07$; $p < 0.01$). No significant changes in larval density were instead associated with the "LdMNPV later" thesis (Figure 1). A significant dose-dependent effect was observed in the trial conducted in 2018 ($F_{12,79} = 19.13$; $p < 0.01$). A higher larval density reduction was associated with a higher LdMNPV dose, and this decrease became more significant as time advanced (Figure 2).

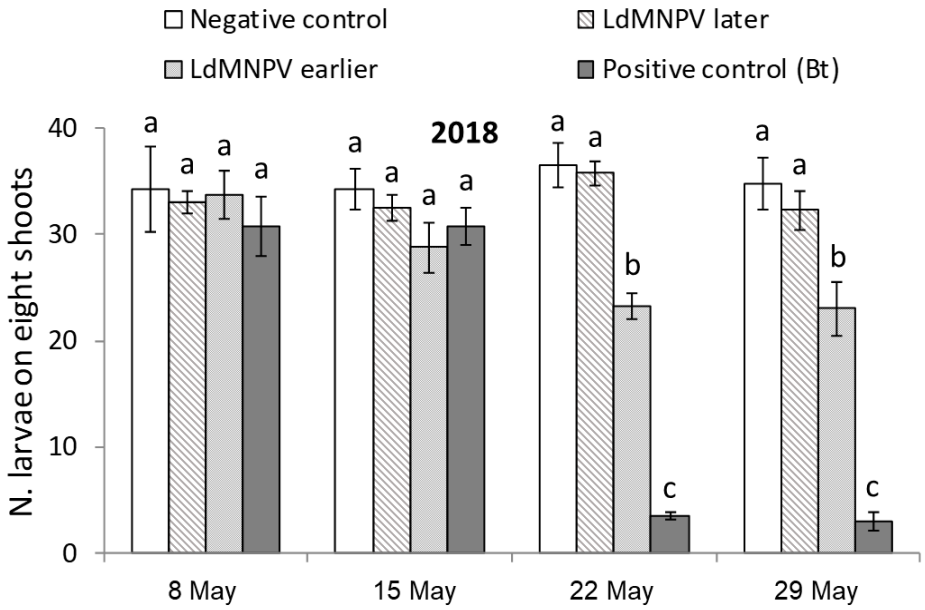

Figure 1. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

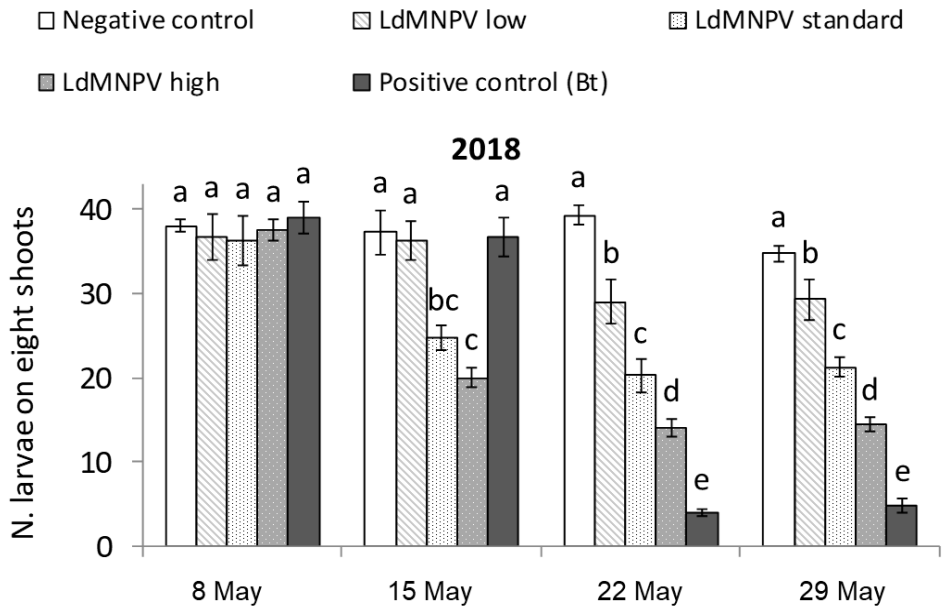

Figure 2. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c, d, e) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

These results were comparable to the output of trials conducted in 2019. In the case of the time–response trial, a significant larval density decrease was achieved by both early and later applications of the baculovirus ($F_{9,63} = 16.62$; $p < 0.01$), with a higher and faster effect of the earlier treatment in comparison to the untreated check (Figure 3). A good efficacy was also observed in the dose–response trial, in which the LdMNPV doses assayed showed a significant biocontrol action on gypsy moth larvae with a dose-dependent effect, in comparison with the untreated control ($F_{12,79} = 9.43$; $p < 0.01$). A greater protection of trees was associated with the highest doses applied (Figure 4).

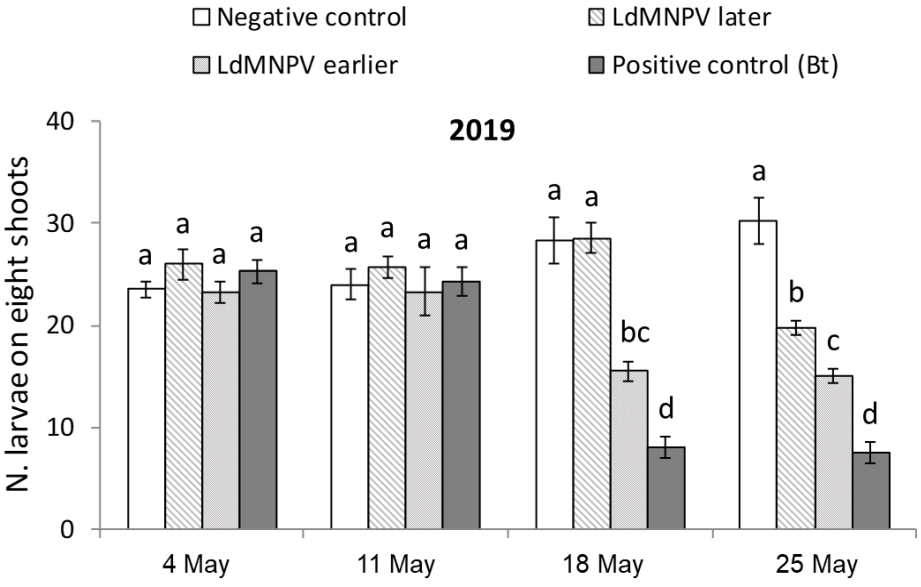

Figure 3. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c, d) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

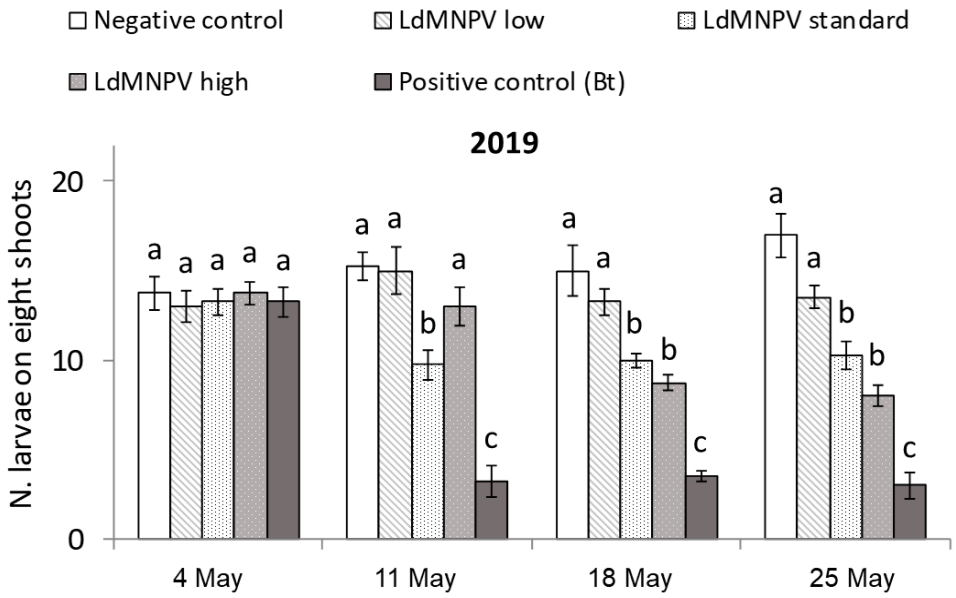

Figure 4. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

In general, a higher percentage of defoliation was found in the untreated check, while a significant protection was associated with higher LdMNPV doses (2018: $F_{4,19} = 24.50$; $p < 0.001$; 2019: $F_{4,19} = 15.43$; $p < 0.01$) and earlier treatments (2018: $F_{3,15} = 33.08$; $p < 0.01$; 2019: $F_{3,15} = 47.61$; $p < 0.01$) (Figure 5).

In all trials, the decrease in larval density and the protection against defoliation in plots treated with the *Btk* reference product was the best and associated with greater and faster action (Figures 1–5).

3.2. Aerial Applications

LdMNPV formulation applied at a dose of 2 L/ha appeared to be well and homogeneously distributed in the treated plots.

In 2019, a general drop in larval density during the season was observed in the experimental area involved in the aerial application study, outlining a retrogradation phase of gypsy moth population in this forest ecosystem in Sardinia. Accordingly, such a reduction was observed in all plots, with no differences among treatments 7 ($F_{2,8} = 3.52$, $p = 0.13$), 14 ($F_{2,8} = 0.95$, $p = 0.46$), and 21 ($F_{2,8} = 1.85$, $p = 0.27$) days after applications (Table 3).

Table 3. Percentage (mean ± SE) of larval density reduction in the field at different time intervals after bioinsecticidal application, in respect to pre-treatment. Percentage data are corrected using the Schneider-Orelli formula.

Days [a]	Treatment			F	*p*
	Foray 76B	LdMNPV	Untreated Check		
7	64.40 ± 8.82 [b]	31.71 ± 4.00	39.66 ± 17.02	3.52	0.13
14	70.28 ± 8.94	34.34 ± 34.33	46.87 ± 18.57	0.95	0.46
21	75.30 ± 9.11	45.59 ± 5.83	50.58 ± 20.39	1.85	0.27

[a] Days after application. [b] No significant differences among means were observed (ANOVA, $p > 0.05$).

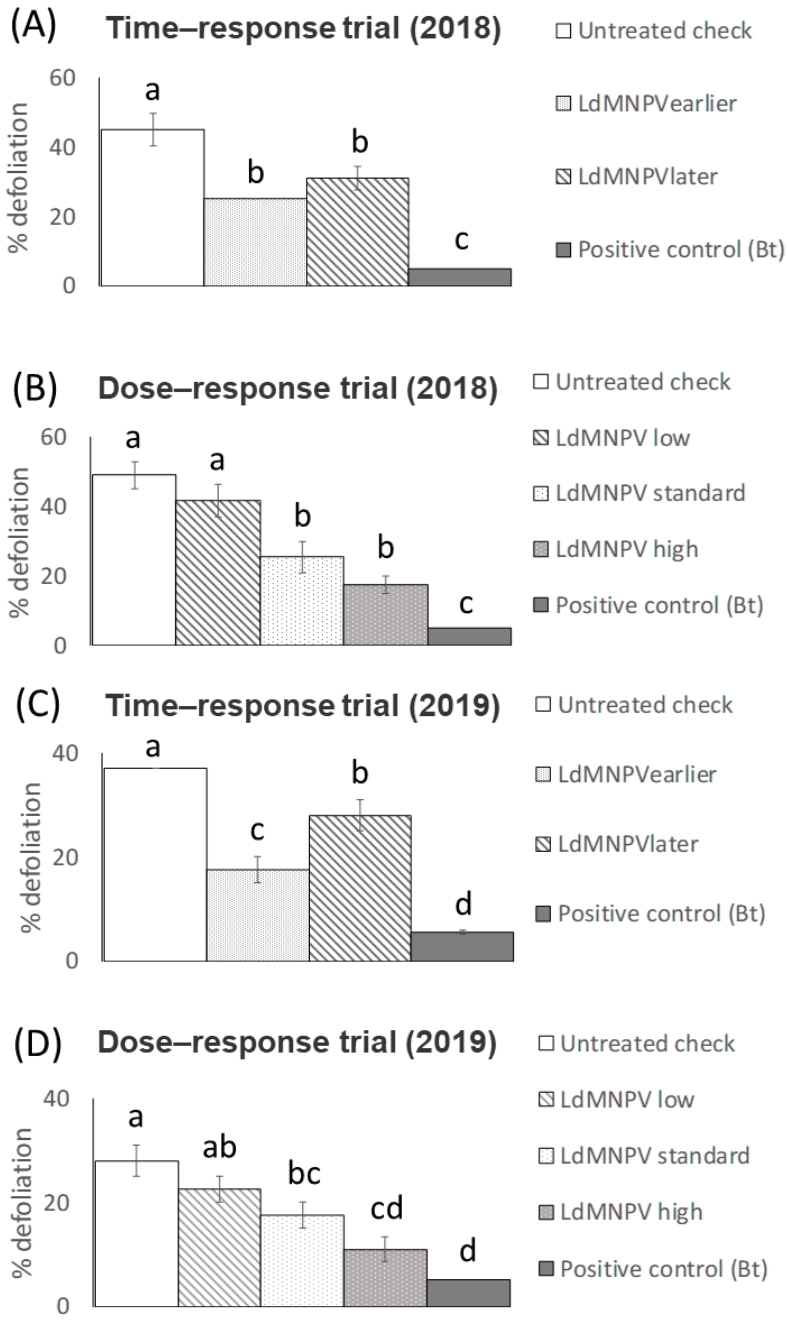

Figure 5. Defoliation percentage (mean ± SE) in different plots treated with LdMNPV from the ground in 2018 (**A**,**B**) and 2019 (**C**,**D**). Different letters (a, b, c, d) above bars indicate significant differences among means (ANOVA, Tukey test, $p < 0.05$).

On the other hand, significant differences in survival rate were observed in the laboratory on the field-collected larvae from different plots ($\chi^2 = 486.79$, $p < 0.01$), with a significant reduction associated with larvae from plots treated with either LdMNPV or *Btk* (Figure 6). In more detail, the survival rate achieved at the end of the observation period was higher for LdMNPV (12%) than *Btk*-treated larvae (0.7%) ($z = -15.73$, $p < 0.01$). The highest survival rate (52%) was instead associated with larvae from untreated plots ($z = -9.08$, $p < 0.01$). The reduction in surviving larvae attributable exclusively to *Btk* and LdMNPV was significantly different between these formulations, either 7 ($t = 7.16$, $p < 0.01$) and 14 ($t = 13.44$, $p < 0.01$) days after applications. Instead, no statistical differences in

corrected larval reduction were found between *Btk* and LdMNPV, 21 days after application (t = 2.75, $p = 0.10$) (Table 4).

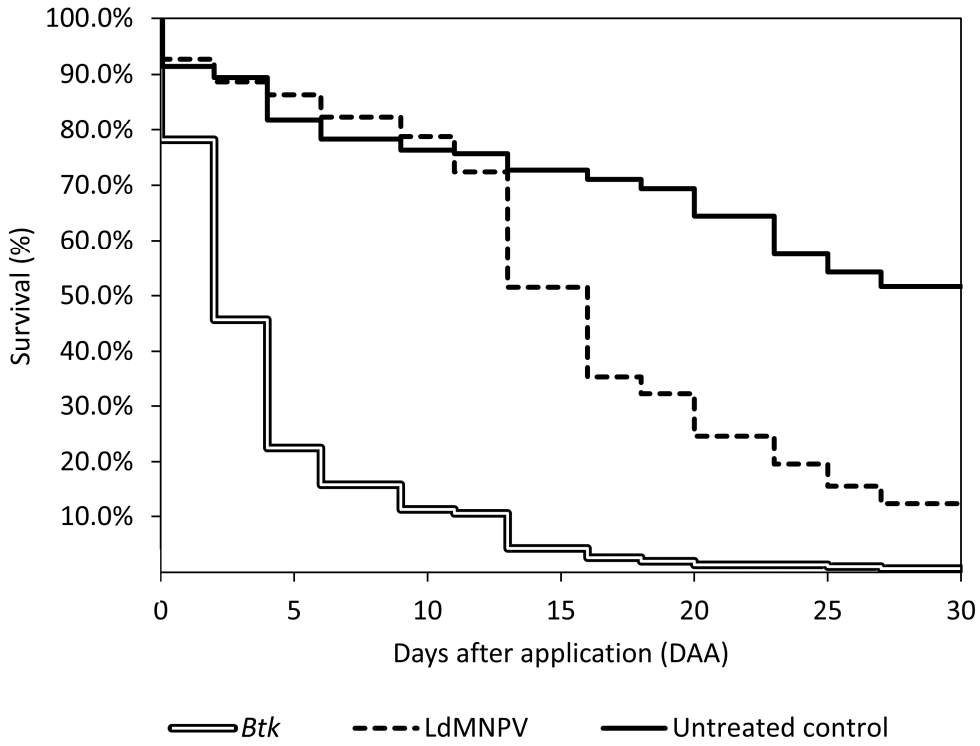

Figure 6. Survival rate of field-collected *Lymantria dispar* larvae from plots treated with Foray76B, LdMNPV formulation, or untreated (control).

Table 4. Reduction percentage (mean ± SE) of surviving larvae in the laboratory attributable exclusively to treatments at different time intervals from bioinsecticidal application.

Days [a]	Treatment [b]		t	*p*
	Foray 76B	LdMNPV		
7	75.44 ± 4.55 [a]	10.05 ± 2.67 [b]	7.16	0.004
14	86.74 ± 1.80 [a]	21.94 ± 2.12 [b]	13.44	<0.001
21	96.33 ± 0.41 [a]	70.19 ± 5.47 [a]	2.75	0.010

[a] Days after application. [b] Different letters in each line indicate significantly different means (Student's *t*-test, $p < 0.05$).

4. Discussion

Baculoviruses represent natural and selective bioinsecticides and have successfully been used against several Lepidopteran pests worldwide. However, their use is limited to niche contexts, due to their narrow host range, a delayed insecticidal action in respect to synthetic chemicals, and economical issues related to industrial production technologies still necessarily relying on the use of living insects as substrates for virus replication [18].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) formulation used in this study showed good efficacy against gypsy moth larval populations in Sardinian forest areas, where this pest is the cause of important defoliations during its periodic outbreaks [19]. In the experiments conducted with applications from the ground, the lethal effects were dose- and time-dependent, with a higher efficacy achieved with higher doses and earlier treatments. These results align with a pathogenic process that begins with the ingestion of occlusion bodies (OBs) releasing occlusion-derived viruses (ODVs) that act in the midgut, infecting epithelial cells [20]. Accordingly, a stronger and faster effect is

expected as a consequence of the earlier ingestion of a higher number of viral particles [21]. It follows that in order to ensure baculovirus' short-time effectiveness, an early application in the season, possibly against the first instar larvae, is of primary importance.

While a good baculovirus efficacy was achieved in these experiments, larval mortality was significantly lower in comparison with plots treated with *Btk*, which was confirmed to be a powerful bioinsecticide against gypsy moth [22,23].

Higher scale experiments involving larger areas and aerial applications of the bioinsecticidal products employing standard doses (2 L/ha) confirmed a reduced survival rate of baculovirus-treated larvae, in respect to the untreated control. Additionally, in this case, *Btk* treatments generated a higher lethal-effect. This greater knock-down power relates to the mechanism of action of solubilized and activated bacterial crystal toxins (Cry proteins) interacting with and disrupting midgut epithelial cells, which leads to insect paralysis and death [24]. This direct toxicity caused by *Btk* is in antithesis with a slower action of the baculovirus depending on an infectious process involving replication of the virus and its spread within the insect body through the tracheal system [20]. Everything considered, a milder action of the virus compared to *Btk* clearly emerged in field trials. Despite such differences, larval population density in 2019 was affected by a natural reduction associated with all treated and untreated plots and related to gypsy moth population retrogradation in Sardinian forest. Accordingly, a more evident efficacy of baculovirus applications in large areas is expected during population progradation, when the baculovirus can express its full potential as a natural regulator of moth population dynamics [25]. Thus, a higher host density triggers horizontal transmission processes, determining a greater number of infected individuals [26]. While these microparasites can naturally regulate periodic cycles of host abundance, their artificial introduction in the forest ecosystem by early applications in the season would produce a similar effect, under appropriate density dynamic conditions. Such density-dependent containment ability has also been demonstrated in laboratory experiments, in which different degrees of resistance to the baculovirus were associated with diverse larval densities [27].

Besides an action normally contained during the season of application, the virus introduced into the forest environment is expected to produce an additional impact on the following generations as a result of sub-lethal effects and vertical transmission [28]. This expectation supports the use of baculovirus against gypsy moth even if the efficacy in the application season is limited. Following an integrated approach to forest management, baculovirus with a slow action, but a detectable midterm impact on subsequent generations, could be combined with applications of *Btk* that generate a more immediate knockdown effect. However, such an emerging hypothesis needs specific multiyear studies to be appropriately documented.

On the other hand, however, it is important that the application of these microbiological control agents is calibrated on the basis of the actual conditions of population dynamics at a given time, in order to produce the desired pest containment effects and make these low-environmental impact interventions even more economically viable. Thus, gypsy moth baculoviruses are good candidates to be introduced in gypsy moth multi-year management programs aiming at interfering with their natural population dynamics.

5. Conclusions

Based on the obtained results in small-scale trials, *L. dispar* showed a significant susceptibility to the LdMNPV formulation, when applied at higher doses and against younger larvae. The highest dose achieved a good efficacy in protecting the crop, albeit at a lower degree than the *Btk* reference product. Such efficacy was not confirmed in larger-scale trials conducted by aerial applications, partly due to population dynamics affected by a natural retrogradation phase. However, a significant increased mortality of larvae collected in plots treated with the baculovirus was detected. Given a higher susceptibility of younger larvae, earlier applications are recommended.

Everything considered, the use of LdMNPV in forest protection programs against the gypsy moth is worth further consideration under different infestation conditions. Its efficacy in regulating population dynamics during outbreaks is expected to be maximized under progradation [29]. This ecological effect could be exploited in a multi-year integrated program involving the combined use of *Btk* to contain infestations and of the baculovirus to modulate population dynamics.

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