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EVALUATION OF DIFFERENT *BACILLUS THURINGIENSIS* SV *KURSTAKI* FORMULATIONS AGAINST *LYMANTRIA DISPAR* AND *MALACOSOMA NEUSTRIA* LARVAE INFESTING *QUERCUS SUBER* TREES ([1])

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Ruiu L., Mannu R., Falchi G., Braggio A., Luciano P. – Evaluation of different *Bacillus thuringiensis* sv *kurstaki* formulations against *Lymantria dispar* and *Malacosoma neustria* larvae infesting *Quercus suber* trees

Lepidopteran defoliators such as *Lymantria dispar* and *Malacosoma neustria* represent a major concern for cork oak forest, especially during population outbreaks. To contain their infestations over large areas, the use of entomopathogenic microorganisms such as *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) is one of the available option. However, the features of the microbial control agent formulations represent a key factor for the success of application programs. The results of two years efficacy trials with different formulations of *Btk*, conducted in 2012 and 2013 in a cork oak forest in North-Western Sardinia, are reported. In the first year, trials were carried out on a *M. neustria* population, while in the second year a mixed population of *L. dispar* and *M. neustria* was involved. Trials included two formulations of *Btk* strain EG 2348 (Rapax® and Rapax Experimental) in comparison with two other commercial formulations (Foray 48B® and Delfin®).

Both formulations of *Btk* strain EG 2348 proved to be effective in controlling the two pest species, showing a forest protection potential comparable to that of the reference products, Foray 48B® and Delfin®, containing spores and insecticidal Cry proteins of strains HD-1 and SA-11, respectively. Also the defoliation levels were significantly higher in untreated control trees than in treated ones.

KEY WORDS: Insect defoliators, entomopathogenic bacteria, cork oak forest, microbial control, efficacy trials.

INTRODUCTION

Lepidopteran defoliators represent a major concern for cork oak forest, especially during population outbreaks that may lead to the complete defoliation of *Quercus suber* L. trees.

In Sardinia (Italy), significant defoliations have over time been caused by larvae of *Lymantria dispar* (L.) (Lepidoptera: Erebidae) and *Malacosoma neustria* (L.) (Lepidoptera: Lasiocampidae) (LUCIANO and PROTA, 1995; LUCIANO and LENTINI, 2007).

These univoltine species have a similar life cycle, overwinter in egg masses, and their larvae hatch from eggs in spring.

L. dispar, also known as gypsy moth, represents a significant risk for the forest in different parts of the world and its cyclically occurring infestations can have an important economical impact. In addition, since its larvae are polyphagous, during outbreaks, defoliation can affect various types of trees (LEONARD, 1981).

M. neustria larvae, also known as tent caterpillars, feed gregariously and gather on plant foliage to construct white webbings (tents) at major branch forks, while later-instar larvae are solitary and feed all over the crown (VERDINELLI *et al.*, 2004).

Cork-oak forest preservation requires the implementation of appropriate management programs to contain the development of insect defoliator populations, especially during outbreaks.

Several pest management methods have over time been considered against Lepidopteran defoliators (WEBB *et al.*, 1998). These include the release of natural enemies (i.e. parasitoids) (WIEBER *et al.*, 1995), the use of broad spectrum insecticides such as diflubenzuron (BERRY *et al.*, 1993), mating disruption techniques employing sexual pheromone dispensers (THORPE *et al.*, 2006) and application of formulations containing entomopathogenic microorganisms like nucleopolyhedroviruses (REARDON and PODGWAITE, 1994), fungi (PILARSKA *et al.*, 2006) and bacteria (GLARE and O'CALLAGHAN, 2000). However, due to biological and practical limitations of these alternatives, to contain defoliator infestations over large areas the use of entomopathogenic microorganisms such as *Bacillus thuringiensis* Berliner serovar *kurstaki* (*Btk*) is the presently available option. *Btk*-based formulations against Lepidoptera usually consist of a mixture including bacterial spores and parasporal bodies (i.e. crystals) containing insecticidal toxins (Cry proteins). After being ingested, solubilised and activated, these proteins binds to specific plasma membrane receptors on the insect midgut epithelium, insert into the cell membrane and determine the formation of amphilic pores. A subsequent abnormal flux of ions and water into the epithelial cell leads to cell

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lysis (ELLAR *et al.*, 1990; BOUCIAS and PENDLAND, 1998). This sequence of histopathological events leads larvae to paralysis and death often associated to bacterial septicaemia (CRICKMORE, 2006).

In order to perform their insecticidal action, spores and insecticidal proteins need to be ingested at a sufficient dose by young larvae (GLARE and O'CALLAGHAN, 2000), so that the efficacy of *Btk* applications in the forest is strictly dependent on the intrinsic features of the formulation (SATINDER *et al.*, 2006) and the way they are applied. An adequate distribution and coverage of target foliage represents a key factor for the success of application programs and the arising of insect resistance can be avoided by the rotation or combination of different bioinsecticidal formulations based on different microbial strains (SMITLEY and DAVIS, 1993; MARTIN and BONNEAU, 2006).

The results of two years efficacy trials with different formulations of *Btk* conducted in 2012 and 2013 in a cork oak forest in North-Western Sardinia against *L. dispar* and *M. neustria*, are reported. The aim of the present work was to compare the efficacy of different *Btk* formulations to protect cork oak trees from caterpillars. The trials were conducted in compliance with Good Experimental Practice (GEP) guidelines established by the European and Mediterranean Plant Protection Organization (EPPO PP 1/210(1), ref. Efficacy evaluation of insecticides – Defoliators of forest trees).

MATERIALS AND METHODS

MICROBIAL FORMULATIONS AND APPLIED RATES

Two formulations of *Btk* strain EG 2348 were tested in two different seasons (2012 and 2013): a commercially available Suspension Concentrate (Rapax®) and an experimental Aqueous Flowable formulation (Rapax Experimental), both from CBC (Europe) Srl, Italy. Both formulations were evaluated in comparison to two commercial *Btk*-based reference products, respectively Foray 48B® (Valent Bioscience Corporation) and Delfin® (Certis USA), and an untreated control. The two *Btk* strain EG 2348-based formulations were tested at two different application rates (respectively 1.0 and 1.5 l/ha), while the *Btk*-based reference products were applied at the recommended label rates (Table 1).

EXPERIMENTAL DESIGN AND ASSESSMENTS

The trials were conducted in a cork oak forest nearby Ploaghe-Chiaramonti (North-Western Sardinia, Italy) in two consecutive spring periods in 2012 and 2013. In the first year (2012) trials were carried out on a *M. neustria* population, while in the second year (2013) a mixed population including *L. dispar* and *M. neustria* was involved.

The actual presence of *M. neustria* and of *L. dispar* in the study areas was verified the previous winter via monitoring and counts of egg masses.

The experimental design consisted in a completely randomized block design with 4 replicates per treatment (plot size: 1 tree). All cork oak trees used in the trial were uniform in size (approximately 5 m in height and with 7 m foliage projection diameter), and showed a comparable initial infestation level.

Before bioinsecticidal applications, the larval density was estimated in each plot following different methods for the two insect species. In the case of *M. neustria*, because of the gregarious behaviour of early-instar larvae, the estimation was initially based on counting the total number of larvae in tents and in groups on branches. Later on, when larvae switched to a solitary behaviour on foliage, larval density was estimated by counting the number of larvae on eight 30 cm-long outer branches, randomly selected on each tree. In the case of *L. dispar*, given the solitary behaviour of all larval instars, before and after treatment application, estimations were based on larval counts on randomly sampled outer branches, as described above for *M. neustria* counting.

Treatments were applied on 11 May in 2012 and on 14 May in 2013, when the majority of larvae were in an early developmental stage (almost exclusively 2nd-3rd-instar larvae) using a motorized knapsack sprayer for experimental trials (M3 series, Cifarelli SpA, Italy).

To estimate the efficacy of the different treatments, insect sampling and counts were performed just before treatment and 1 and 2 weeks after application. Furthermore, 2 weeks after treatment application, defoliation caused by *L. dispar* and/or *M. neustria* larvae was estimated by assigning percent defoliation values according to the Guidelines for Evaluation of Crown in the Mediterranean Region (Economou *et al.*, 1994) including the following *Quercus suber* L. specific scale: 5%, 15%, 35%, 50%, 75%.

STATISTICAL ANALYSIS

The number of larvae/tree (preliminary assessment on *M. neustria* gregarious larvae), the number of larvae/8 branches at each assessment and the percentage of defoliation at the final assessment were compared across treatments using 1-way ANOVA followed by LSD test to separate means in each sampling interval.

Table 1 – Tested treatments and applied rates.

Treatment	Active ingredient ingredient (strain)	Concentration a.i. (%)	Formulation[1]	Applied rate
Untreated Control	–	–	–	–
Rapax®	*Btk* EG2348	7.5	SC	1.5 l/ha
Rapax®	*Btk* EG2348	7.5	SC	1.0 l/ha
Rapax Exp.	*Btk* EG2348	7.5	AF	1.5 l/ha
Rapax Exp.	*Btk* EG2348	7.5	AF	1.0 l/ha
Delfin®	*Btk* SA11	6.4	WG	750 g/ha
Foray 48B®	*Btk* HD1	2.1	AF	3.0 l/ha

[1] SC, Suspension Concentrate; AF, Aqueous Flowable Formulation; WG, Water Dispersible Granule.

RESULTS

At the preliminary assessment, just before treatment application, no significant differences among treatments in the number of larvae per tree emerged. The initial larval infestation level was thus comparable among treatments. The mean total number of *M. neustria* larvae/tree ranged from 104.5 to 247.5 in 2012 and from 132.75 to 293.50 in 2013, with differences among treatments not being significant (2012: $F = 2.51$, $df = 6$, $P = 0.0547$; 2013: $F = 0.79$, $df = 6$, $P = 0.5673$). Also, in the case of *L. dispar* in 2013, the initial infestation level was comparable among treatments. The mean number of larvae on 8 branches/tree ranged from 0.50 to 2.25, with no significant differences among treatments ($F = 0.75$, $df = 6$, $P = 0.6129$).

All tested products proved to be effective against *L. dispar* and *M. neustria* larvae under open field conditions: all tested treatments significantly reduced the number of both lepidopteran species in comparison to the untreated control. Significant differences in the number of larvae/8 branches per tree among treatments compared to the untreated control were observed for *M. neustria* one and two weeks after treatment application in 2012 (after one week: $F = 37.94$, $df = 6$, $P < 0.0001$; after two weeks: $F = 105.46$, $df = 6$, $P < 0.0001$) (Table 2) and in 2013 (after one week: $F = 6.60$, $df = 6$, $P = 0.0005$; after two weeks: $F = 8.23$, $df = 6$, $P = 0.0001$) (Table 3), and for *L. dispar* one ($F = 5.74$, $df = 6$, $P = 0.0012$) and two weeks ($F = 8.13$, $df = 6$, $P = 0.0001$) after treatment application in 2013 (Table 4).

Table 2 – Number (m ± SE) of *M. neustria* larvae/8 branches in the tested treatments at the different post-treatment assessments in 2012*.

Treatment	N. larvae on 8 branches /tree	
	18 May	25 May
Untreated control	17.8 ± 0.8 a	22.3 ± 1.4 a
Rapax® (1.5 l/ha)	2.0 ± 0.6 c	2.0 ± 0.4 c
Rapax® (1.0 l/ha)	5.3 ± 1.3 b	2.5 ± 0.7 c
Rapax Exp. (1.5 l/ha)	1.3 ± 0.6 c	1.8 ± 0.5 c
Rapax Exp. (1.0 l/ha)	5.5 ± 1.7 b	5.3 ± 0.9 b
Delfin®	3.0 ± 0.4 bc	2.5 ± 0.3 c
Foray 48B®	2.0 ± 0.4 c	1.8 ± 0.3 c

*Means in the same column followed by different letters are significantly different (1 way ANOVA followed by LSD test: P<0.05).

Table 3 – Number (m ± SE) of *M. neustria* larvae/8 branches in the tested treatments at the different post-treatment assessments in 2013*.

Treatment	N. larvae on 8 branches /tree	
	21 May	28 May
Untreated control	41.75 ± 13.92 a	49.25 ± 14.68 a
Rapax® (1.5 l/ha)	5.25 ± 2.02 b	8.50 ± 2.33 b
Rapax® (1.0 l/ha)	4.25 ± 2.25 b	3.00 ± 0.71 b
Rapax Exp. (1.5 l/ha)	13.50 ± 3.48 b	8.75 ± 3.01 b
Rapax Exp. (1.0 l/ha)	3.25 ± 0.85 b	4.50 ± 1.44 b
Delfin®	1.75 ± 0.85 b	4.25 ± 0.63 b
Foray 48B®	3.25 ± 1.31 b	5.75 ± 0.75 b

*Means in the same column followed by different letters are significantly different (1 way ANOVA followed by LSD test: P<0.05).

Table 4 – Number (m ± SE) of *L. dispar* larvae/8 branches in the tested treatments at the different post-treatment assessments in 2013*.

Treatment	N. larvae on 8 branches /tree	
	21 May	28 May
Untreated control	3.75 ± 0.95 a	8.00 ± 2.48 a
Rapax® (1.5 l/ha)	0.50 ± 0.29 b	0.25 ± 0.25 b
Rapax® (1.0 l/ha)	1.50 ± 0.65 b	0.50 ± 0.50 b
Rapax Exp. (1.5 l/ha)	0.25 ± 0.25 b	1.00 ± 0.41 b
Rapax Exp. (1.0 l/ha)	1.00 ± 0.41 b	1.00 ± 0.41 b
Delfin®	0.50 ± 0.29 b	0.25 ± 0.25 b
Foray 48B®	0.75 ± 0.25 b	0.25 ± 0.25 b

*Means in the same column followed by different letters are significantly different (1 way ANOVA, followed by LSD test: P<0.05).

Except for Rapax Experimental at the lower rate (1.0 l/ha), the efficacy in reducing the number of *L. dispar* and *M. neustria* larvae of *Btk* strain EG 2348 was always comparable to that of the *Btk*-based reference products in both years.

Percent defoliation levels 2 weeks after treatment application, were significantly higher in untreated control plots than in treated plots in both years (2012: $F = 72.42$, $df = 6$, $P < 0.0001$; 2013: $F = 68.16$, $df = 6$, $P < 0.0001$), with differences among treated plots not being significant in 2012. In 2013, instead, % defoliation values were not always comparable among treated plots (Table 5). Mean percent defoliation according to the Guidelines for Evaluation of Crown in the Mediterranean Region in the untreated trees reached 75%, while it was below 10% for Rapax® at both rates, Foray 48B® and Delfin® in both years. Significantly higher defoliation values than for the former treatments were recorded for Rapax Experimental at 1.0 l/ha (mean: 17.5%), while intermediate values were observed for Rapax Experimental at 1.5 l/ha (mean: 12.5%).

DISCUSSION AND CONCLUSION

In our experimental conditions all formulations ensured a significant protection of cork oak foliage compared to the untreated control.

The lower efficacy and the significant dose-response effect observed for the Rapax Experimental at the lower

Table 5 – Percent defoliation (m ± SE) caused by the two lepidopteran species in the tested treatments at the different post-treatment assessments in 2012 and 2013*.

Treatment	Defoliation (%)	
	2012	2013
Untreated control	75.0 ± 0.0 a	75.0 ± 0.0 a
Rapax® (1.5 l/ha)	5.0 ± 0.0 b	7.5 ± 2.5 c
Rapax® (1.0 l/ha)	5.0 ± 0.0 b	7.5 ± 2.5 c
Rapax Exp. (1.5 l/ha)	10.0 ± 2.9 b	12.5 ± 2.5 bc
Rapax Exp. (1.0 l/ha)	12.5 ± 7.5 b	17.5 ± 6.3 b
Delfin®	5.0 ± 0.0 b	7.5 ± 2.5 c
Foray 48B®	5.0 ± 0.0 b	5.0 ± 0.0 c

*Means in the same column followed by different letters are significantly different (1 way ANOVA, followed by LSD test: P<0.05).

rate in 2012 could be associated to a slight though not significantly higher mean initial infestation level observed on trees treated with this aqueous flowable formulation. On the other side, it is known that the effectiveness of *Bt*-based products is dose dependent and a higher efficacy is achieved increasing application dosages (GLARE and O'CALLAGHAN, 2000).

According to these results, all formulations of *Btk* strain EG 2348 seem to be effective in protecting cork oak trees from *L. dispar* and *M. neustria* defoliations. The susceptibility of these insect species to *Btk* is documented by more than 10 year-data from trials involving aerial applications with Foray 48B formulations against Sardinian populations of lepidopteran defoliators (LENTINI and LUCIANO, 1995; LUCIANO and LENTINI, 2007). In our experiments involving insecticidal applications from the ground, no significant differences in larval mortality between Rapax® and Rapax Experimental emerged when formulations were applied at the higher dosage (1.5 l/ha). However, differences in insecticidal performance could be expected with aerial applications where the size and density of droplets is a major concern (SATINDER *et al.*, 2006). In these application conditions a high efficacy of aqueous flowable formulations (AF) has been reported (RUIU *et al.*, 2012). With the purpose to maximize efficacy in field conditions, different formulations have over time been developed by the industry (LORD, 2005). In a recent study, LADURNER *et al.* (2011) investigated the efficacy of different formulations of *Btk* strain EG 2348 against the tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), on tomato, and in their studies the suspension concentrate proved to be more effective than the wettable powder. Under our trial conditions, the suspension concentrate (Rapax®) and the aqueous flowable (Rapax Experimental) formulations of *Btk* strain EG2348, applied as a broadcast foliar spray from the ground, showed comparable and high efficacy against *L. dispar* and *M. neustria*. Similar efficacy was obtained with the formulations containing strains HD-1 (Foray 48B® a.i.) and SA-11 (Delfin® a.i.). All tested strains produce crystal proteins belonging to Cry1 and Cry2 families. Strain EG2348 expresses proteins Cry1Aa, Cry1Ac, Cry2A; strain HD-1 expresses proteins Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry2B; and strain SA-11 expresses proteins Cry1Ab e Cry1Ac.

The availability of a variety of *Btk* strains expressing different Cry toxins is of great importance to ensure mode of action variability, which can contribute to prevent the possible development of insect adaptations (GRIFFITTS and AROIAN, 2005). According to this concept, the alternation of different bioinsecticidal formulations based on diverse microbial strains or different active ingredients, associated to the maintenance of *Bt*-sensitive populations in treatment-free areas, should be considered from a resistance management perspective.

Btk-based formulations have been successfully used to control gypsy moth and tent caterpillars (VAN DER LAAN and WASSINK, 1962; SMITLEY and DAVIS, 1993; LUCIANO and LENTINI, 2007). However, further research is continuously needed to evaluate improved formulations and to investigate the possible effect of application method (e.g. broadcast foliar spray versus aerial spray) and equipment on the efficacy of the formulated products.

Since aerial spraying is considered the most valuable application method of *Btk*-based products for the control of forest defoliators, further studies on the efficacy of *Btk* strain EG2348 applied via aerial spraying are deemed necessary.

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RIASSUNTO

VALUTAZIONE DI DIFFERENTI FORMULAZIONI DI *BACILLUS THURINGIENSIS* SV *KURSTAKI* PER IL CONTROLLO LARVALE DI *LYMANTRIA DISPAR* E *MALACOSOMA NEUSTRIA* SU ALBERI DI *QUERCUS SUBER*

Lepidotteri defogliatori come *Lymantria dispar* e *Malacosoma neustria* costituiscono un importante rischio per le foreste di quercia da sughero, specialmente durante le fasi di culmine della popolazione. L'impiego dei microrganismi entomopatogeni come *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) per il contenimento delle loro infestazioni su superfici estese rappresenta una delle poche soluzioni attualmente disponibili. Tuttavia, le caratteristiche delle formulazioni a base di agenti di controllo microbiologico costituiscono un fattore chiave per il successo dei programmi di intervento. Il presente lavoro riporta i risultati di due anni di prove sperimentali di efficacia condotte nel 2012 e 2013 mediante differenti formulazioni di *Btk* in una sughereta della Sardegna nord-occidentale. Nel primo anno, le prove sono state condotte su una popolazione di *M. neustria*, mentre nel secondo anno su una popolazione mista di *L. dispar* e *M. neustria*. Le sperimentazioni hanno incluso due formulazioni di *Btk* ceppo EG 2348 (Rapax® e Rapax Experimental) in comparazione con due formulazioni commerciali di riferimento (Foray 48B® and Delfin®).

Entrambe le formulazioni di *Btk* ceppo EG 2348 si sono mostrate efficaci nel controllo delle due specie di lepidotteri, evidenziando un medesimo potenziale di protezione della foresta in comparazione con i prodotti di riferimento, Foray 48B® e Delfin®, contenenti spore e proteine insetticide Cry dei ceppi HD-1 e SA-11, rispettivamente. Anche i livelli di defogliazione sono risultati sempre maggiori nel controllo non trattato rispetto alle piante diversamente trattate.

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