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PROPHYLACTIC EFFECT OF ABITYLGUANIDE IN NUCLEAR POLYHEDROSIS OF SILKWORM

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ABSTRACT

Abitylguanide, an effective antiviral against DNA-containing viruses pathogenic for humans, manifested a marked activity toward the baculovirus-induced nuclear polyhedrosis of the silkworm, Bombixmori L. Applied at experimental conditions during the spring and autumn silkworm feedings, 0.1–0.15% abitylguanide solution strongly decreased (> 9–10 times) the incidence of spontaneous polyhedrosis. An increase in cocoon yield (by 3%)and quality was also registered. These effects were confirmed during trials carried out in two consecutive years at the industrial scale. We found a substantial increase in cocoon production in both spring (by 3–5%) and autumn (by about 14%), which was markedly higher. These results were obtained by spraying a 0.1% abitylguanide aqueous solution on mulberry leaves. Cocoon quality was improved compared to a dry control group.

Keywords: Abitylguanide, antiviral activity, nuclear polyedrosis, silkworm, cocoon production

INTRODUCTION

Nuclear polyhedrosis (grasserie) of the silkworm (*Bombixmori* Linnaeus) is a disease caused by a DNA-containing virus, the nuclear polyhedrosis virus BmNPV, a member of the *Baculoviridae* family, which includes rod-like viruses in insects (Fenner, 1976). A characteristic peculiarity of this virus is the formation of polyheders, crystal protein inclusions containing numerous virions, in the nuclei of the stunned cells (Kawarabata et al., 1980). The affected cells are in the hypodermis, fat tissue, tracheal matrix, and hemolymph. This usually happens during the last larval stage of development, toward the end of the fifth instar, and quickly leads to silkworm larvae becoming yellow and swollen followed by destruction due to tissue softening and lysis. Acute BmNPV infection kills silkworms and spreads the disease throughout their colony. Subacute infection leads to pupal death and formation of inferior quality cocoons (Savanurmathet al., 1994).

The spreading of grasserie is due to the BmNPVpolyhedrals, which are highly stable structures that persist for a long time in the environment. Regularly found on the surface of plants or in the soil, they are resistant to deterioration and are sufficiently stable to persist from season to season (Torquatoet al., 2006; SoriGobena and Bhaskar, 2015).

Infection is transmitted vertically (transovarially) and horizontally (virus on the egg surface) and has a latent character (Tarasevich, 1975). The disease appears when ecological standards of silkworm breeding are destroyed. Most deviations in ecological factors as well as other stress factors that decrease insect vitality – such as temperature increase or decrease, insufficient aeration, increased humidity, inadequate nutrition, poor food quality (wet or withered mulberry leaves), and increased density of progeny – induce spontaneous polyhedrosis(Ovanessian, 1973). Researchers have proven that spontaneous polyhedrosisresults from activation of the latent infection (Aruga, 1963; Tarasevich, 1975). Thus, latency underlies viral regulation of the insect population (Kullyev et al., 1990).

Nuclear polyhedrosis in silkworms is a widely spread disease causing significant damages in the sericulture industry. For example,it is responsible for 70–80% of the industry's financial losses in India (Basavarajappa, 1993; Savanurmathet al., 1994) and over 50% of the total losses in Bulgaria (Manchev, 1979).

In view of the viral etiology of the disease and the absence of effective immunological means for protecting the insects, the struggle with nuclear polyhedrosis continues embarrassed. Attempts to influence the disease by chemical means date back to the 1950s. It was established that folic acid, N-aminobenzoic acid, vitamin B_{12}, and Co^{++} enhance resistance to the development of grasserie through their stimulating effect on silkworm growth and development without any activity against BmNPV (Tarasevich, 1975; Tarasevich and Ulanova, 1965). Other nucleic acid antimetabolites, such as aminopterin (folic acid analogue) and 2,6-diaminopurine, suppress the disease course but, at the same time, delay silkworm development. In addition, these substances applied at lower doses induce spontaneous polyhedrosis(Tarasevich, 1965). Glutamic acid, which inhibits the tobacco mosaic virus and a *Streptococcus lactis* phage, suppresses the disease course, but it also delays caterpillar growth (Tarasevich and Ulanova, 1965). In fact, means, preparatives, and methods for etiotropic prevention and therapy are absent.

The markedly manifested antiviral activity of abitylguanide against certain DNA-containing viruses – adenoviruses (Galabov et al., 2017) and herpesviruses (Galabov, Vilaginès, 1970; Galabov, 1978) – directed us to test the activity of this compound against *Baculovirusbombicis*BmNPV.

Here, we present the results of our tests of abitylguanide's effect on the development of spontaneous nuclear polyhedrosis in silkworms bred (i) in experimental conditions at the Sericulture Experimental StationinVratza, Bulgaria, and (ii) in normal field conditions atthe Sericulture Department of Industrial Agrarian Complex in Harmanli, Haskovo District, Bulgaria.

MATERIALS AND METHODS

Silkworms *Bombixmori* L. of the hybrid J-124 x C-122 were hatched and grown at optimal conditions. After the second molt, silkworms were fed with mulberry leaves sprayed with an aqueous solution of 0.1–0.15% abitylguanide immediately before feeding. The treatment was continued uninterrupted until silkworms moved to the bushes, a duration of 15–18 days.

The following experimental groups of 300 silkworms each (3 repetitions, 100 silkworms per repetition) were tested: (i) silkworms fed abitylguanide-sprayed leaves once daily; (ii) two times daily; (iii) at each feeding, (i.e., 4–5 times daily); (iv) control – silkworms fed water-sprayed leaves once daily; (v) silkworms fed water-sprayed leaves at each feeding; (vi) “dry” control (feeding with fresh, untreated mulberry leaves). The experiments were conducted twice during two consecutive years.

Silkworm development was observed by measuring their food intake and by recording the appearance of sick or dead worms. Sick or dead worms were removed and studied by microscopy (at 1200 X) for the presence of polyhedral bodies or nosema (pebrine) spores. The doubtful ones were placed in a humid chamber (for mycoses).

In a second-stage, production-scale (field) trial, the following *Bombixmori* L. hybrids were used: Shingetsu x Hosho in the spring feeding; Hebr 1 x Hebr 2 (Bulgarian hybrid) in the summer feeding; and Kinshu x Showa in the autumn feeding. This stage was also conducted over two consecutive years.

During the first year, three seasons of silkworm feedings (in Bulgaria, bivoltine to trivoltine silkworm breeding is carried out) took place – spring, summer, and autumn – and four experimental treatments were applied: (i) 0.5% abitylguanide in an aqueous solution; (ii) 0.1% abitylguanide in an aqueous solution); (iii) wet control (mulberry leaves treated with water before feeding); (iv) “dry” control (feeding with fresh, untreated mulberry leaves). In the autumn feeding, an additional experimental treatment was included: 0.05% abitylguanide in an aqueous solution. A separate capsule of silkworms was used for each experimental treatment. For the spring feeding, a private house was used as the rearing house, and in the summer and autumn feedings, one of the silkworm breeding rooms at the Industrial Agrarian Complex in the town of Harmanli was used. For the first two instars, silkworms were grown in the mechanized lines following the scheme of triple feeding overnight and preserving the required climate conditions. The indicated experimental schemes were established at the onset of the third age. During the entire larval stage, the triple feeding overnight and the accepted climate regime were strictly preserved. Some deviation was allowed during the spring feeding: during the last two days, silkworms were fed only two times daily. The following indexes of silkworm growing were recorded: (i) total cocoon yield per capsule; (ii) cocoon regular quality distribution of the yield – quality I + II, couples, quality III.

During the following year, the trial was carried out in the spring and in the autumn silkworm breedings. This trial used only two silkworm feeding treatments: (i) 0.1% abitylguanide in aqueous solutionat each feeding,beginning the firstday after the second sleep through Day 6 of the 5thinstar; (ii) dry control. The magnitude of the trial was substantially increased. The total number of capsules was 120, divided into two trial groups (two silkworm breeding rooms with 30 capsules each).As in the previous year, indexes of silkworm growth were recorded, as were raw cocoon weight (g) cocoon silk coat weight (mg), and raw cocoon silkiness (%).

Constant microscopic control for the presence of polyhedral bodies or nosema (pebrine) spores in the sick and dead worms during the production-scale silkworm feeding trials was carried out.

RESULTS

Experimental study

Observations of larval development found that the silkworms consumed theabitylguanide-sprayedmulberry leaves. Within the usual larval stage of 25–26 days, without showing any signs of disease, the silkworms rose in unison to the bushes, and their cocoons were normal in size and with undamaged silk fibers.

In the untreated groups (no abitylguanide), the first silkworms with nuclear polyhedrosis appeared at the end of the 5th age, Day 7–8, and others appeared after the silkworms rose to the bushes (Day 9).

Tables 1 and 2 show that silkworms in the abitylguanide-treated groups produced significantly more cocoons compared to the control groups. In the early spring growth (May, in two successive years; Table 1), significantly less spontaneous polyhedrosis was seen in theabitylguanide-treated groups, and the percentages of cocooned silkworms in those groups were 89.2% with a single application, 92.7% with two applications daily, and 96.7% with 4–5 applications daily. In contrast, the control group with a single wetting had 73.3% cocooned silkworms and the control group with repeated wettings had 63.6%. A significant difference in cocooning was seen between groups with repeated wettings (a provoking factor) – 96.7% in the presence of abitylguanide and 63.6% in the control (without the compound). The difference with the control group that had a single wetting was also significant, even though during the early spring feeding, silkworms have an increased resistance to spontaneous nuclear polyhedrosis: only 73.3% cocooned with single wetting of the food in the control condition, and the application of a stronger provocation (4-fold) led to an increased morbidity rate for nuclear polyhedrosis.

In experiments carried out with the treatment conditions of the late spring growing (June; Table 2), a significantly higher percentage of spontaneous polyhedrosis was observed in the control groups (as is usual). In this case, a single daily wetting of the food was a strong provoking factor for the increase of polyhedrosis mortality: from 13.1% in the “dry” control to 18.8% in the control with a single wetting.

The single application of abitylguanide manifested a marked protective effect: 89.8% of silkworms cocooned when treated with 0.1% compound solution and 97.6% when treated with 0.15% solution, whereas in the control group (water only) only 66.5% cocooned. The percentage dead via polyhedrosis was strongly reduced – from 18.8% in the wet control group to 5.1% in the group treated with 0.1% abitylguanide and 2.1% in the group treated with 0.15% abitylguanide.

It should also be noted that the silkworms’ general resistance to infections was obviously increased in abitylguanide-treated groups. For example, the percentage of silkworms dead of bacterial flacherie was on average 4.8–7.7% in the controls, 2.5% in the 0.1%abitylguanide group, and 0 in the 0.15% abitylguanide group.

Silkworm production (field) trial

The second stage could be described as trials of abitylguanide's effect on silkworm breeding at the industrial (field) scale. These trials were carried out in two consecutive years. The compound was applied at each feeding with mulberry leaves.

Results of Year I field tests (Table 3) demonstrated that treatment with 0.1% abitylguanide during all three seasons and treatment with 0.05% abitylguanide in the autumn produced higher average cocoon yields compared to the control feeding schemes. These differences were 11.0–15.3% larger during the spring and autumn feedings, which are periods with more illnesses.

Trials on the effect of abitylguanide treatment on silkworm breeding were carried out on a significantly larger scale during Year II. Testing schemes were simplified, applying 0.1% abitylguanide in aqueous solution at each feeding of mulberry leaves (treatment scheme) to 60 capsules of silkworms, and feeding fresh, untreated mulberry leaves ("dry" control) to silkworms in another 60 capsules. These trials were conducted on silkworm feedings during the spring (Tables 4 and 5) and autumn (Table 4).

Abitylguanide applied at 0.1% aqueous solution markedly increased cocoon yields compared to controls, both the total yield and the yield of regular-quality cocoons. During the spring silkworm feeding, the increases were 2.8% and 5%, respectively. These percentages were markedly higher for the autumn silkworm feeding, where the total cocoon yield per capsule increased 13.8% and the yield of regular-quality cocoons increased 10.8%, compared to controls.

Regarding cocoon quality, the data for the spring feedings also demonstrated a favorable effect of the abitylguanide treatment compared to the control group (Table 5): raw cocoon weight was increased by 2.6%, silk coat weight was increased by 4.2%, and raw cocoon silkiness was increased by about 2%.

These data, in agreement with the experimental study's results, are undoubtedly due to a prophylactic effect of abitylguanide on the development of spontaneous nuclear polyhedrosis in growing silkworms.

The abitylguanide effect is strongly dependent on the concentration used. It is evident that the 5-times higher concentration of abitylguanide exerts an unfavorable effect on silkworm growing (Table 3).

It is well known that the increased humidity during the spring silkworm feeding is a factor that contributes to the appearance of spontaneous grasserie. Despite the fact that the compound was applied as an aqueous solution, which could be considered an unfavorable approach, it manifested a marked protective effect against the development of the disease. However, the invention of a device for application of abitylguanide as a micronized powder on mulberry leaves could be beneficial, especially during the spring silkworm feeding.

DISCUSSION

In general, the viral diseases of *Bombixmori* are difficult to manage due to the very short life cycle of silkworms (Khyade and Tyagi, 2017).

The described experimental study on the effect of abitylguanide on spontaneous nuclear polyhedrosis in silkworms demonstrated that: (i) a 0.1% abitylguanide solution applied during the early spring silkworm feeding strongly diminished, more than 10 times, the incidence of spontaneous polyhedrosis; (ii) during the late spring silkworm feeding, the protective effect of the compound at 0.1–0.15% concentration was also very well expressed, with a 9-fold decrease in the disease at 0.15% concentration; (iii) the 3% increase in cocoon yields during the autumn silkworm feeding could be due to the liquid form of compound application, which irrigated the mulberry leaves; (iv) regarding raw cocoon quality, the increase in yields was mainly due to first and second quality cocoons (the basic cocoon categories of interest in the industry); (v) applied at a 0.1% aqueous solution,abitylguanide is harmless to silkworms.

It appears that the antiviral abitylguanide possesses a pronounced protective action against the spontaneous form of silkworm nuclear polyhedrosis. The compound's effect is most likely due to inhibition of BmNPV replication. A strong argument for this connection is the fact that multiple applications of abitylguanide every day (at each feeding) led to the most strongly expressed protection (96.7% cocooned), despite the fact that multiple wettings is a strong provoking factor for nuclear polyhedrosis during the early spring feeding. Our results showed that only 63.6% of the silkworms cocooned in the control group with multiple wettings and 73.3% in the control group with a single wetting.

These effects were convincingly confirmed during the industrial-scale trials. The compound's inhibitory effect on BmNPV replication showed a marked protective activity against the development of spontaneous grasserie. A substantial increase in cocoon productionwas found, regardless of the season. Production increased by 3–5% in the spring and was markedly higher, by about 14%, in the autumn. These results were obtained through the application (spraying) of a 0.1% abitylguanide aqueous solution on the mulberry leaves. In addition, cocoon quality was improved as compared to the dry control group.

The Introduction section mentioned studies done in the past "classic" period, but here we discuss more recent investigations into preventive and curative agents and approachesin the struggles against grasserie. First, we highlight the results obtained by applying *Spirulinaplatensis*, a multicellular filamentous blue-green alga (cyanobacterium; Mahesh Babuet al., 2005). This study was carried out on silkworms experimentally infected with BmNPV obtained from crystals. *S. platensis* applied daily as a 10% suspension on *Bombixmori* larvae during the third instar, via the mulberry leaves, manifested a marked protective effect: a 90% resistance to BmNPV.

Another prospective approach would be the involvement of antiviral microRNAs. Researchers established that the cytoplasmic immune system process was stimulated in silkworms infected with another*Bombixmori*–attacking agent, the cytoplasmic polyhedrosis virus (BmCPV; Wu et al., 2013).
In addition, an anti-BmNPV related gene and protein have been found in the silkworm (*Bombixmori*). These structures increased silkworm resistance to BmNPV, which could lead to alleviation and elimination of grasserie (Gaoet al., 2013).

We must also mention the studies on the development of an oral “vaccine” against nuclear polyhedrosis based on obtaining attenuated BmNPV (Natarajuet al., 2001). Triple vaccination could protect silkworms for a comparatively long period, decreasing mortality due to nuclear polyhedrosis by 85.12% (Hui-Penget al., 2007).

Thus, several positive achievementshave been made regarding efforts to involve stimulation of the silkworm immune system in the resistance to grasserie development.

The data presented here convincingly manifested a marked effectiveness of abitylguanide against BmNPV-induced spontaneous nuclear polyhedrosis. With these results and the data on *Spirulinaplatensis*activity as an anti-BmNPVagent, these substances, these inhibitors of BmNPV replication, could be considered the first antivirals against grasserie.

In summary, our results favor the use of chemotherapy against BmNPV as a promising approach in the struggle against grasserie.

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Table 1. Effect of abitylguanide on spontaneous nuclear polyhedrosis in silkworm hybrid J-124 x C-122 at early spring growth.

Experimental group	Cocooned %	Dead %	
		Nuclear polyhedrosis	Other diseases
Abitylguanide	$89.2 \pm 1.8^{+++}$	$9.3 \pm 1.7^{++}$	1.5
Abitylguanide 0.1% solution twice daily	$92.7 \pm 1.0^{+++}$	$6.1 \pm 1.2^{++}$	1.2
Abitylguanide 0.1% solution at each feeding (4–5 times daily)	$96.7 \pm 1.3^{+++}$	$2.6 \pm 0.7^{+}$	0.7
Control single wetting	$73.3 \pm 1.1^{+++}$	$20.0 \pm 0.9^{+++}$	6.7
Control wetting at each feeding (4–5 times daily)	$63.6 \pm 1.1^{+++}$	$26.6 \pm 1.2^{+++}$	9.8

*Note.*Each experimental group contained a total of 600 silkworms (3 repetitions × 100 silkworms per repetition, performed twice). The percentage cocooned is based on the average number of cocoons in each experimental group.Sick or dead silkworms were immediately removed and examined under a light microscope for polyhedral bodies (nuclear polyhedrosis) and nosema spores (nosematosis), and incubated in a humid chamber – for mycoses.

+ $p < .05$; ++ $p < .01$; +++ $p < .001$

Table 2. Effect of abitylguanide on spontaneous nuclear polyhedrosis in silkworm hybrid J-124 x C-122 at late spring growth.

Experimental group	Cocooned %	Dead %	
		Nuclear polyhedrosis	Other diseases
Abitylguanide 0.1% solution once daily	89.8±2.1^{+++}	5.1±0.9^{++}	5.1
Abitylguanide 0.15% solution once daily	97.6±1.0^{+++}	2.1±0.8^{++}	0.3
Control single wetting	66.5±1.8^{+++}	18.8±1.0^{+++}	14.7
“Dry” control	81.3±2.3^{+++}	13.1±0.9^{+++}	5.6

Note. Each experimental group contained a total of 600 silkworms (3 repetitions × 100 silkworms per repetition, performed twice). The percentage cocooned was based on the average number of cocoons in each section. Sick or dead silkworms were immediately removed and examined under a light microscope for polyhedral bodies (nuclear polyhedrosis) and nosema spores (nosematosis), and incubated in a humid chamber – for mycoses.
+ $p < .05$; ++ $p < .01$; +++ $p < .001$

Table 3.Production trial of abitylguanide during silkworm feeding in stable-type silkworm breeding rooms during Year I.

Trial schemes	Total yield per capsule, kg	Regular quality I+II, kg*	Couples, kg	Quality III, kg
Spring				
Abitylguanide 0.5%	18.56	15.57 (14.85 + 0.82)	0.37	2.62
Abitylguanide 0.1%	27.04	23.31 (21.93 + 1.38)	0.49	3.24
Wet Control	24.1	18.89 (18.05 + 0.84)	0.75	4.34
Dry Control	23.35	20.96 (19.47 + 1.49)	0.66	2.73
Summer				
Abitylguanide 0.5%	12.3	9.49 (8.46 + 1.03)	0.37	2.35
Abitylguanide 0.1%	19.6	15.58 (14.19 + 1.39)	0.49	3.49
Wet Control	19.4	15.24 (13.98 + 1.26)	0.75	3.36

Dry Control	16.7	12.93 (12.46 + 0.47)	0.66	3.4
Autumn				
Abitylguanide 0.5%	18.75	16.28 (15.4 + 0.88)	0.39	2.08
Abitylguanide 0.1%	19.8	16.95 (16.69 + 0.26)	0.44	2.42
Abitylguanide 0.05%	20.7	17.91 (17.35 + 0.56)	0.6	2.1
Wet Control	17.05	14.54 (13.66 + 0.88)	0.57	2.84
Dry Control	16.5	16.35 (15.28 + 1.07)	0.3	1.85

[*] Total cocoon yields for the first and second qualities are given separately in brackets.

Table 4.Production trial of abitylguanide during silkworm feeding in stable-type silkworm breeding rooms during Year II.

Trial schemes	Total yield per capsule, kg	Regular quality I+II,kg	Couples, kg	Quality III, kg
Spring				
Abitylguanide 0.1%	21.15	19.49	1.29	0.37
Control	20.56	18.53	1.61	0.42
Autumn				
Abitylguanide 0.1%	29.0	26.5	1.33	0.9
Control	25.0	23.65	0.94	0.64

Note. Abitylguanide was applied in an aqueous solution at each feeding beginning the first day after the second sleep through Day 6 of the 5th age. The total number of capsules was 120: two trial groups, each with two silkworm rearing rooms, with 30 capsules per room.

Table 5.Production trial of abitylguanide during silkworm feeding in stable-type silkworm breeding rooms during the spring of Year II.

Trial schemes	Total yield per capsule, kg	Regular quality I+II, kg	Couples, kg	Quality III, kg	Raw cocoon weight, g	Silk coat, weight, mg	Raw cocoon silkiness, %
Abitylguanide 0.1%	21.15	19.49	1.29	0.37	1.515	306	20.2
Control	20.56	18.53	1.61	0.42	1.475	293	19.83