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Efficacy of a neem cake for the control of *Culicoides* biting midges larvae

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Abstract

The larvicidal efficacy of a commercial neem cake containing 3,750 ppm of azadirachtin A+B, 7,980 ppm of salannin and 1,850 ppm of nimbin was assayed in laboratory and in field trials for the control of *Culicoides* larvae. Laboratory bioassays were conducted with neem cake on larval mortality of *Culicoides* in water after 7 days and a lethal concentration value (LC_{50}) of 0.37 g/l was obtained. A neem cake methanol extract was separated by different solvents and fractions of increasing polarity were assayed on *Culicoides* larvae. The most active ethyl acetate fraction containing 1 ppm of azadirachtin, 1.5 ppm salannin and 0.3 ppm of nimbin was more toxic than a commercial formulation at the same azadirachtin concentration.

A treatment with neem cake at dose of 100 g/m^2 was applied in a larval breeding site of *Culicoides* located in a riverside of a pond margin of a livestock farm in Sardinia, Italy. The emergence of *Culicoides* adults in treated and untreated plots was observed weekly using emergence traps before and after treatment. In plots treated with the neem cake, a significant reduction in *Culicoides* emergence was recorded until 28 days. *Culicoides imicola*, the main vector of Bluetongue Virus, represented about 10% of all emerged *Culicoides* adults and resulted highly sensitive to the neem cake.

KEY WORDS: CULICOIDES LARVAE, BLUETONGUE VECTORS, NEEM CAKE, AZADIRACHTIN, SALANNIN

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Introduction

In the last decade multiple epidemics of Bluetongue occurred in Sardinia (Italy) resulting in the loss of >500,000 sheep (1). In the Mediterranean basin, the principal vector of Bluetongue Virus (BTV) is *Culicoides imicola* Kieffer (Diptera: Ceratopogonidae), although other species belonging to the *C. obsoletus* and *C. pulicaris* species complexes are suspected vectors, too. The direct control of adult vectors through the application of insecticides in infected holdings and onto the back of the animals appeared ineffective, while a mass vaccination campaign resulted in a significant reduction in clinical cases of the disease (1). No efforts were made to control *Culicoides* larvae because the breeding sites of *C. imicola* were poorly identified and characterized. Recent studies in Sardinia demonstrated that *C. imicola* is found in muddy habitats lacking surface water, such as those found adjacent to leaking watering troughs and along pond margins contaminated with animal faeces (1; 2).

The control of *Culicoides* larvae have been attempted mainly against species responsible for biting nuisance rather than arbovirus transmission; however some experiments to assess the efficacy of larvicides have been carried out on the North American BTV vector *Culicoides variipennis* (Coquillett) (3).

In the last years, the insecticidal effect of the extracts from seed kernels of the Indian neem tree, Azadirachta indica, and of the more active ingredient, the nortriterpene azadirachtin (Az), have been found active against many livestock pests (4). The aim of this study was to evaluate the efficacy of a neem cake product against *Culicoides* larvae in laboratory and in field.

Methods

A commercial neem cake in pellet form, GreeNeem Cake (NeemGreen, Virudhunagar, India), obtained as a by-product from the neem oil extraction process from kernels and seeds of *Azadirachta indica*, was used as test material against *Culicoides* larvae. The chemical analysis of the GreeNeem Cake methanol extract showed the presence of azadirachtin A (2,750±100 ppm) and B (1,000±15 ppm), salannin (7,980±50 ppm), nimbin (1,850±100 ppm) and several polycyclic aromatic substances (5). The neem cake methanol extract was also submitted to a fractionation process and HPLC analyses for nortriterpenes contents and fractions of increasing polarity (Hp, hexane fraction; Ep, ethyl acetate fraction; Bp, butanol fraction; Wp, aqueous fraction) were tested on *Culicoides* larvae. The HPLC analysis of Ep solution (50 ppm of extract) revealed the presence of Az_A (0.7 ppm), Az_B (0.3 ppm), salannin (1.5 ppm) and nimbin (0.3 ppm) (5).

Culicoides larvae for laboratory test were obtained by sieving mud samples taken from margins of an artificial pond. Only second and third instars larvae were exposed to serial doses of neem cake mixed in tap water, whereas neem cake fractions were tested at 50 ppm concentration. The toxicity of the most active solution Ep (Az_A+B 1 ppm) was compared with a commercial azadirachtin formulation (Oikos 25 Plus, Az_A+ B 25 g/l) at 1, 10 and 100 ppm Az concentration. Four replicates of 10 larvae for treated samples and untreated control were set up into plastic jars containing 60 ml of the test solutions and mortality was evaluated after 7 days.

Field trials were conducted in a pond margin of a livestock farm near Sassari (Italy), where a preliminary sampling showed a large population of *Culicoides* larvae. The soil surface was treated with GreeNeem Cake at the dose of 100 g/m^2 on 7 October 2008. A randomized complete block design with 4 replicates of treated and untreated plots of 1 m^2 surface (2 m along shoreline x 0.5 m) was assessed along the higher-lying pond margin. A mud sample from each plot was taken weekly before and until 28 days after treatment to estimate the population of *Culicoides* larvae. Each mud sample of about 800 cm^3 was scraped from the soil surface at 20 cm above shoreline using a flat trowel and maintained in laboratory for 30 days for retrieval of emerging biting midges (1).

Dose-mortality data from laboratory bioassays were analysed by probit analysis (6). Corrected percent mortality of neem cake fractions and of the Az based formulation was calculated using Abbott's formula. To analyze the differences among treatments in field trials, data of emerged adults belonging to different *Culicoides* species were compared using a repeated measures analysis of variance (ANOVA), performed with the Generalized Linear Mixed Model (GLMM) procedure. A one-way ANOVA was applied to compare the emergences of *Culicoides* adults before treatments. The Tukey's test was used to separate significantly different means. All statistical analysis were conducted with the SAS for Windows (7) with the significance level set at $\alpha = 0.05$.

Results

Laboratory bioassays

The toxicity of GreeNeem Cake to *Culicoides* larvae was concentration dependent, as confirmed by probit analysis ($x^2 = 35.32$, df = 1, $P < 0.0001$), which showed a LC_{50} and LC_{90} values of 0.37 g/l (95% CL = 0.26-0.48) and 1.19 g/l (95% CL = 0.92-1.82), respectively, and a slope ± standard error (S.E.) of 2.54 ± 0.43.

The neem cake fractions tested at 50 ppm concentration showed a significant mortality only for the Ep (ethyl acetate fraction) and Hp (hexane fraction) ($F_4 = 173.05$, $P = 0.0000$) (Table 1). The Ep fraction which contained 1 ppm of azadirachtin was more toxic to *Culicoides* larvae than the technical azadirachtin solutions at 1 and 10 ppm Az concentration ($F_4 = 215.70$, $P = 0.0000$) (Table 2).

see Table 1.

see Table 2.

Field trials

A total of 1986 biting midges belonging to the family Ceratopogonidae emerged from mud samples taken from control and treated plots during the study period. The most abundant adults belonged to the *Culicoides* genus, that represented 94% of total midges, whereas *Bezzia*, *Forcipomyia* and *Dasyhelea* adults were obtained in low number. *Culicoides imicola*, the main vector of BTV, represented about 10% of all emerged adults.

The number of *Culicoides* emerging before treatment was not significantly different in treated and untreated plots ($F_{1,6} = 0.42$, $P = 0.5410$). One-way repeated measures ANOVA analyses of *Culicoides* adults after the application of GreeNeem Cake showed significant treatment effects ($F_{1, 6} = 43.59$, $P = 0.0006$) and significant interaction effects between treatment and sampling date ($F_{3, 18} = 5.74$, $P = 0.0061$). The treatment determined a significant reduction of *Culicoides* density for 28 days (Figure 1).

The most abundant emerging species, *C. cataneii*, *C. circumscriptus*, *C. festivipennis* and *C. imicola*, appeared to be very sensitive to the treatment with GreeNeem Cake (Table 3).

see Fig. 1

see Table 3.

Discussion

The experiments described in this paper showed that a neem cake product showed a significant effect on larvae of *Culicoides* in laboratory and field tests. This neem by-product after the industrial treatment still contains relevant quantities of nortriterpenes, that are biologically active on insects (5; 8). The main nortriterpene component found in this product was salannin, followed by azadirachtin and nimbin.

Azadirachtin is considered the most important active principle contained in neem seed kernels, and its efficacy was already evaluated for the control of *Culicoides* larvae (9). However in our tests a commercial formulation of azadirachtin was less effective than an ethyl acetate fraction (Ep) of neem cake that contained the same concentration of Az in addition to salannin and nimbin. Therefore, the larvicidal effectiveness of neem cake could be

related to the complexity of its composition with respect to a commercial neem product based on isolated azadirachtin.

Our field trials revealed the possibility of using neem cake for the control of *Culicoides* larvae on breeding sites. This product is an effective larvicide and remain active for a long period of time, showing a considerable residual effect for a month. Moreover, herbal products containing azadirachtin also proved to have oviposition deterrent and ovicidal effects on *Culicoides* midges (9).

Culicoides larvae breed in muddy environments rich in organic matter, such as artificial and natural ponds, and moist habitats nearby animal drinking stations. The development of cheap and safe larvicides as the neem cake should aid in the improvement of vector control programs. However, the neem cake composition depends on the quality of seeds as well as on the extraction processes used. For its application in control programs it is therefore necessary to obtain reliable information about the composition in nortriterpenes of the different types of commercial neem cake on the market (8)

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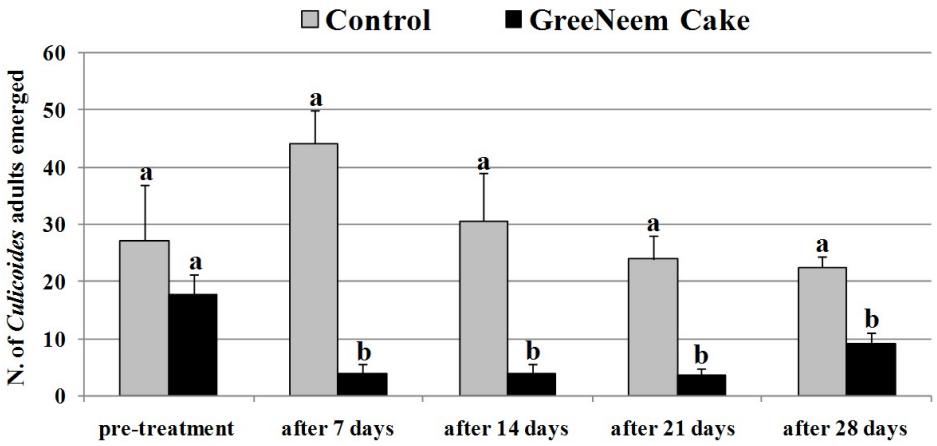

Figure 1. Mean number (± S.E.) of *Culicoides* adults emerging from mud samples taken weekly in control and treated plots in Sassari (Sardinia, Italy) during October 2008 (means in pre-treatment followed by the same letter are not significantly different by ANOVA at the 5% level; means after treatment followed by the same letter are not significantly different by one-way repeated measures ANOVA at the 5% level).

Treatment	Mean mortality (% ± SD) [a]	% corrected mortality [b]
Control (H_2O)	2.50 ± 5.00 a	
Hp	90.00 ± 0.00 b	89.74
Ep	97.50 ± 5.00 b	97.44
Bp	2.50 ± 5.00 a	0.00
Wp	10.00 ± 14.14 a	7.69

Table 1. Comparison of the activity of neem cake fractions on *Culicoides* larval mortality after 7 days.
[a] Means followed by different letters are significantly different.
[b] Percent larval mortality was calculated using the Abbott's formula.

Treatment	Mean mortality (% ± SD) [a]	% corrected mortality [b]
Control (H_2O)	2.50 ± 5.00 a	
Oikos 25 Plus (Az 1 ppm)	2.50 ± 5.00 a	0.00
Oikos 25 Plus (Az 10 ppm)	20.00 ± 8.16 b	17.95
Oikos 25 Plus (Az 100 ppm)	90.00 ± 8.16 c	89.74
Ep (Az 1 ppm)	97.50 ± 5.00 c	97.44

Table 2. Comparison of the activity of Ep fraction and Az commercial formulation at various concentrations on *Culicoides* larval mortality after 7 days.
[a] Means followed by different letters are significantly different.
[b] Percent larval mortality was calculated using the Abbott's formula.

Treatment	*C. cataneii*	*C. circumscriptus*	*C. festivipennis*	*C. imicola*
Control	52.71 ± 12.45 a	21.29 ± 3.01 a	32.38 ± 8.95 a	12.29 ± 2.23 a
GreeNeem Cake	4.63 ± 1.55 b	4.08 ± 1.82 b	6.88 ± 2.89 b	1.75 ± 0.66 b
P	*0.0018*	*0.0066*	*0.0433*	*0.0009*
F (1, 6 df)	*28.27*	*16.58*	*6.52*	*36.59*

Table 3. Mean number (± S.E.) of adults of *C. cataneii*, *C. circumscriptus*, *C. festivipennis* and *C. imicola* emerging during 4 weeks from mud samples in control and treated plots at Sassari (Sardinia, Italy) during October 2008 (means followed by the same letter within a column are not significantly different by one-way repeated measures ANOVA at the 5% level).