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RAPID COMMUNICATION

To bee or not to bee: creating DNA insecticides to replace non-selective organophosphate insecticides for use against the soft scale insect *Ceroplastes japonicus* Green

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Abstract

Obviously, the moment has come in agriculture and forestry when we must decide to gradually abandon (where possible) non-selectively acting chemical insecticides, taking into consideration the overall decrease in the total biomass of insects, especially pollinators, and the increased number of diseases and human deaths directly or indirectly associated with chemical insecticides. Yet with the world facing the rapid growth of human populations, the annual reduction of cultivated areas, and substantial losses from insect pests, most experts believe that no serious alternative to chemical insecticides exists. However, there is definitely room to create more well-tailored chemical insecticides. And there is hope, in the form of effective DNA insecticides able to provide an adequate level of safety for non-target organisms. In this short communication describing experiments carried out on the larvae of *Ceroplastes japonicus* Green (feeding on *Ilex aquifolium* Linnaeus), we show for the first time the enormous potential for the use of DNA insecticides in the control of soft scale insects and how they could replace non-selective organophosphate insecticides.

Keywords: *Ceroplastes japonicus*, DNA insecticides, insect pest control, 28S ribosomal RNA, soft scale insects

Loss of biodiversity has become a major issue worldwide, and the current rates of species decline – which could progress into extinction – are unprecedented (Barnosky *et al.* 2011). The proportion of insect species currently in decline (41%) is twice as high as that of vertebrates and one of the main drivers is pollution, caused primarily by synthetic pesticides and fertilizers (Sánchez-Bayo and Wyckhuys 2019). A rethinking of current agricultural practices, in particular a serious reduction in pesticide usage and its substitution with more sustainable, ecologically based practices, is urgently needed to slow or reverse current trends

(Gomiero *et al.* 2011; Dudley *et al.* 2017). On the other hand, modern agricultural practices will be difficult to change or abandon. Rapid population growth creates more mouths to feed, while causing less arable land to be available. Cultivated areas face annual reduction and substantial losses from insect pests, leading most experts to agree that there are no serious alternatives to insecticides (Furlan and Kreutzweiser 2015) because they help to preserve 20% of all crops (Oerke 2006). So, we need insecticides. It therefore seems obvious that the production of effective insecticides, safe for non-target organisms and with a short environmental half--life, is the strategically correct solution. Nevertheless, right now preference is still given to the development and use of classes of non-selective chemical insecticides, such as organophosphate (OP) insecticides.

Fenitrothion is a phosphorothioate, one of the most widely used OP insecticides (Adeyinka and Pierre 2020), is known for its acute toxicity toward non-target organisms. At chronic levels, it suppresses the growth of algae and crayfish and is highly toxic to rats (the lowest oral LD_{50} was 240 mg · kg^{-1} of body weight (range 240–1,700 mg · kg^{-1} of body weight) (WHO 2004). Exposure to some OPs has been associated with the possible development of cancer (Adeyinka and Pierre 2020). Fenitrothion residues persist a long time in the environment after application, and are subject to different types of degradation, including photolysis, hydrolysis, and biological degradation (Lacorte and Barcelo 1994; Kadum 2019). Sufficient information exists to characterize fenitrothion as highly toxic to honeybees (Thomson 1982; U.S. Environmental Protection Agency 1987; Kidd and James 1991). Despite the high risk of some chemicals, namely neonicotinoids (Laurino *et al.* 2013), most insecticide residues in pollen and honey present a moderate risk to bees (1–5%), especially pyrethroid and OP insecticides (Sánchez-Bayo and Goka 2016). Unfortunately, cold temperatures make pyrethroid and OP insecticides more toxic to bees (Johansen 1977; Decourtye *et al.* 2003). Moreover, the honey produced in apiaries has been found to be contaminated with organochlorines and organophosphates, usually at higher concentrations, than honey produced by wild *Apis* species (Sarfraz Khan *et al.* 2004). It should go without saying that beneficial insects as important as honeybees cannot be put at even a moderate risk. Honeybees are crucial for both the production of honey and the pollination of crop plants. The European honeybee, *Apis mellifera* L., is an essential pollinator of agricultural crops in many countries, pollinating crops worth $200 billion globally every year (van Engelsdorp and Meixner 2010).

Since most modern chemical insecticides have a relatively long half-life, the concentration of the target chemical agent increases in the ecosystem upon transition from a lower to a higher trophic level. Persistent use of chemical insecticides in agriculture and forestry poisons the participants in all trophic levels of aquatic and terrestrial ecosystems where for most chemical agents (xenobiotics) no enzymes exist to catalyze their rapid decomposition. Therefore, the only safe way to control the number of insect pests is the use of molecules of natural origin that can be safe and effective at the same time (Oberemok *et al.* 2018). The aim of this study was to show that the successful use of DNA insecticides based on unmodified polymers of natural origin (DNA) is possible and how they could replace modern, indiscriminately active insecticides such as organophosphates. In general, two main goals were pursued. First, to show that DNA insecticides have the potential to replace many modern non-selective chemical insecticides and reduce the resultant ecotoxicological burden on ecosystems. Second, to show that DNA insecticides are highly effective against soft scale insects, using the example of Japanese wax scale (*Ceroplastes japonicus* Green).

Scale insects are major agricultural pests (Miller *et al.* 2005) and their economic impact is connected to their ability to hide on all parts of the host plants. The Japanese wax scale *C. japonicus* Green (Hemiptera: Sternorrhyncha: Coccidae) is native to East Asia (China, Japan, and North and South Korea). The species, which is polyphagous, is a pest of soft and hardwood trees, fruit trees, citrus trees, and ornamentals in urban environments and has been observed on more than 100 plant species from 38 different families (Morales *et al.* 2016). The host plants most commonly infested by these insects are *Citrus*, *Diospyros*, *Ilex*, and *Hedera* (Pellizzari and Germain 2010), which means that Japanese wax scale is also a destructive pest in many forests. For our research, the *Ilex aquifolium* L. plant was used, which is pollinated by bees.

This experiment was performed in triplicate between September and October 2019 within the grounds of the Nikita Botanical Garden (Republic of Crimea, Yalta). We designed an 11 nt long antisense oligonucleotide (5′-CGACCGACGAA-3′, CJ-11) from the *C. japonicus* 28S ribosomal RNA gene and applied them to the target plant (1 mg of DNA per m^2 of plant leaves using a concentration of 0.1 g · l^{-1} DNA in a water solution) to compare the insecticidal effect with that of an organophosphate insecticide (fenitrothion, 2 g · l^{-1}). In the groups treated with water, ACGT (control group – 5′-ACGTACGTACG-3′), CJ-11, and fenitrothion, we observed larval deaths of 11.09, 11.09, 29.07, and 89.25%; 14.84, 22.08, 72.26, and 99%; and 17.28, 21.91, 78.82, and 100%, respectively, on the 4th , 7th, and 10th day after treatment (CJ-11 vs. control: $\chi 2 = 39.383$, $p < 0.001$, $N = 800$, $df = 1$; $\chi 2 = 266.712$, $p < 0.001$, $N = 800$, $df = 1$; $\chi 2 = 300.606$, $p < 0.001$, $N = 800$, $df = 1$) (Table 1). The non-parametric Pearson's chi-squared test ($\chi 2$) with Yates's correction were used to

Table 1. Mortality of *Ceroplastes japonicus* larvae (shown as a percentage)

Day	Control	ACGT	CJ-11	Fenitrothion
4th	11.09 ± 12.08	11.09 ± 5.4	29.07 ± 23.81*	89.25 ± 2.21*
7th	14.84 ± 10.76	22.08 ± 9.93	72.26 ± 16.00*	99.00 ± 0.81*
10th	17.28 ± 7.84	21.91 ± 3.59	78.82 ± 18.60*	100.00 ± 0.00*

*significant difference in comparison to the control group ($p < 0.001$); means ± SE are represented in the table

evaluate the significant difference between the groups' means (STATISTICA 7 software, Palo Alto, CA, USA). Fenitrothion also caused significant mortality compared to the control.

The CJ-11 insecticide showed substantial efficacy in comparison to fenitrothion. Given the natural origin of DNA insecticides, this approach can be a significant competition for OP insecticides and other non-selective chemical insecticides.

Total RNA was isolated from *C. japonicus* larvae using ExtractRNA Reagent (Evrogen, Russia) according to the manufacturer's instructions. To produce the replicates for each treatment, three independent extractions were carried out. The quality of the extracted total RNA was assessed by loading 5 µl of the eluted volume onto a 1.8% agarose gel and running the gel in TBE (Tris-Borate-EDTA) buffer (10 V/cm) for 40 min. The quantity, intensity, and pattern of RNA bands were equal in all experimental groups, confirming the quality and reproducibility of the RNA extraction from the insect material. For reverse transcription, the total RNA of *C. japonicus* (0.4 µg) was annealed with CER-R primer (5′-CGAACTGAAAACGCGTCC-3) using an MMLV Reverse Transcriptase kit (Evrogen, Russia) according to the manufacturer's instructions. The cDNA of the insect pests and the following primers, forward 5′-ACAGAGCCCGTGAATCC-3′ and reverse 5′-CGAACTGAAAACGCGTCC-3′ for *C. japonicus,* were used for quantitative real-time PCR studies and amplification with gene specific primers to quantify the *C. japonicus* 28S rRNA. The concentration of the 28S rRNA in insects treated with CJ-11 was significantly lower (3.1 fold) than that of the controls (water-treated) (Fig. 1). 28S and 5.8S rRNAs constitute about 85–90% of total cellular RNA, and are very useful as internal controls (Paule and White 2000). Thus, we provide evidence that the target 28S rRNA is degraded and that the CJ-11 fragment decreases its concentration as an antisense RNase H-dependent oligonucleotide (Dias and Stein 2002). We also detected a notable decrease (1.3 fold) in the concentration of 28S rRNA in the ACGT group. When evaluating the slightly increased mortality rates of larvae in the ACGT groups, we suggest that ACGT may non-specifically regulate the concentration of 28S rRNA. The ACGT fragment contains CpG motifs and these dinucleotides are known to activate

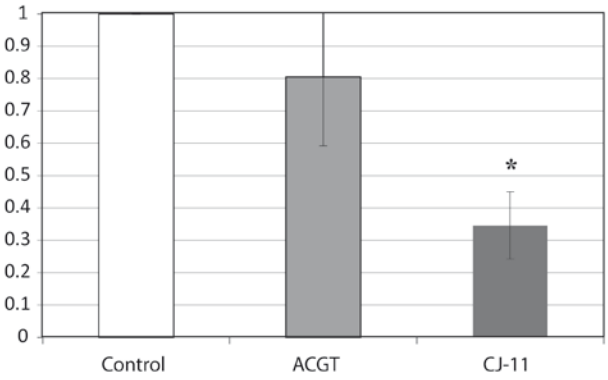

Fig. 1. Relative concentration of 28S rRNA in *Ceroplastes japonicus* 4 days after treatment with the DNA oligonucleotides. Data represent the means and standard errors of ribosomal RNA concentrations for three replicates relative to the control (water-treated) group. Control is taken as 1 (100%)

a host's innate immunity against lethal challenges from a wide variety of pathogens (Krieg 2002). Obviously, CpG motifs are capable of generating 'unexpected' effects (Oberemok *et al.* 2017) and should not be used as a control; however, they do expand our understanding of the action of the oligonucleotides on the insect. In any case, they did not cause significant insect death when used as a part of random ACGT oligonucleotide. Thus, in this experiment, DNA insecticides were for the first time successfully applied to soft scale insects.

To summarize the work, contact DNA insecticides tested for the first time in a series of experiments in 2008 (Oberemok *et al.* 2017) demonstrated their potential in the development of a post genomic approach in agriculture and forestry (Oberemok *et al.* 2018). The ability to create short antisense fragments from the conservative parts of the genes of insect pests opens up the possibility of using this approach in anti-resistance programs. It also provides a method for creating safe and effective DNA insecticides using a universal mechanism, changing only the combination of nitrogenous bases depending on the gene sequences of the target pest.

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