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Conifer flavonoid compounds inhibit detoxification enzymes and synergize insecticides

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ABSTRACT

Detoxification by glutathione S-transferases (GSTs) and esterases are important mechanisms associated with insecticide resistance. Discovery of novel GST and esterase inhibitors from phytochemicals could provide potential new insecticide synergists. Conifer tree species contain flavonoids, such as taxifolin, that inhibit *in vitro* GST activity. The objectives were to test the relative effectiveness of taxifolin as an enzyme inhibitor and as an insecticide synergist in combination with the organophosphorous insecticide, Guthion (50% azinphos-methyl), and the botanical insecticide, pyrethrum, using an insecticide-resistant Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Say) strain. Both taxifolin and its isomer, quercetin, increased the mortality of 1st instar CPB larvae after 48 h when combined with Guthion, but not pyrethrum. Taxifolin had greater *in vitro* esterase inhibition compared with the commonly used esterase inhibitor, S, S, S-tributyl phosphorotrithioate (DEF). An *in vivo* esterase and GST inhibition effect after ingestion of taxifolin was measured, however DEF caused a greater suppression of esterase activity. This study demonstrated that flavonoid compounds have both *in vitro* and *in vivo* esterase inhibition, which is likely responsible for the insecticide synergism observed in insecticide-resistant CPB.

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1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is the most devastating insect pest of the potato. Continuous feeding on potato foliage from larvae to adult causes heavy damage and reduced yield [1]. The use of synthetic insecticides to control this pest has led to resistance in virtually all insecticide classes, including organophosphates (OPs) [2], carbamates, pyrethroids [3] and neonicotinoids [4].

Insecticide-resistant insects typically have higher detoxification rates than those that are susceptible, therefore one strategy is to combine insecticides with synergists to reduce the metabolism. Target site mutations can often be the cause of resistance, but these are not affected by synergists [5]. Synergists are nontoxic alone, but in combination with an insecticide will inhibit the insect's mixed-function oxidases or other detoxification enzyme systems in order to restore the susceptibility to the insecticides. The most common synergists are those that inhibit cytochrome P450 mixed-function oxidases, for examples those developed from natural compounds with a methylenedioxyphenyl moiety, such as sesamin, sesamolin, myristicin and dillapiol [6,7].

The synergist can also be used as a diagnostic tool in resistance management to identify the mechanism potentially involved in resistance [8]. For example, specific enzyme inhibitors such as S, S, S-tributyl phosphorotrithioate (DEF), an esterase inhibitor, piperonyl butoxide (PBO), a cytochrome P450 inhibitor, and diethyl maleate (DEM), a glutathione S-transferase (GST) inhibitor are commonly used to inhibit different detoxification enzymes both the resistant and susceptible strains [4,9]. The testing of several synergists led to the identification of the mechanism involved in a lambda-cyhalothrin resistant strain of lady beetle *Eriopis connexa* (Germar) [10]. The synergism ratio with DEF was 50-fold higher than a susceptible strain indicating esterases played an important role in the resistance [10].

Currently, CPB is ranked among the top 10 insecticide-resistant pests in the world having developed many different resistance mechanisms [11], including enhanced metabolism, target site insensitivity, increased rate of excretion, reduced cuticle penetration and behavioral resistance [4,12–14], with enhanced detoxification enzymes being the most common mechanism. The involvement of the previously mentioned detoxification enzyme classes in CPB insecticide resistance has been confirmed by the use of synergists. For example, esterase-mediated detoxification was important for CPB resistance to the OP, phosalone, as determined with DEF treatments [15]. Similarly, PBO treatments confirmed that monooxygenases are involved in chlorantraniliprole and pyrethrum detoxification [16,17], and that GSTs play a role in the metabolism of the molting hormone agonist diacylhydrazine based on DEM treatments [18].

However, there is evidence that the continuous use of the previously listed synergists has negative mammalian and environmental health consequences [19]. Hence, from both an agrochemical industry and a human and environmental health perspective, there is a need for new insecticide synergist discovery. A rich source of new compounds from

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plants are continuously discovered, many with recognized medicinal activity that also affects insect physiological and biochemical function. In tropical America, plant species within the Piperaceae family are used as a folk medicine [20] and have been promoted more recently as biopesticides because of the insect neurotoxin compounds, piperamides [6] and as synergists because of the neolignans, including dillapiol [17]. In North America, several conifer species from the Pinaceae family have been used to treat the symptoms of diabetes by the Cree of Eeyou Istchee, including Jack pine (*Pinus banksiana* Lamb.), black spruce (*Picea mariana* (Mill.) BSP.), balsam fir (*Abies balsamea* (L.) Mill.) and tamarack larch (*Larix laricina* (Du Roi) Koch) [21], and the wood and bark extracts contain insect growth inhibitory properties [22]. Conifer phenylpropene compounds, such as vanillin and isoeugenol, significantly inhibit the enzyme and receptor sites in the neuroendocrine GABAergic and dopaminergic systems [23], many of which are conserved between mammals and arthropods. These sites are also important targets for the pharmaceutical treatment of many neurological disorders and indicate the potential of novel phytochemical discovery for both medicinal and insecticidal applications. The screening of phytochemicals through *in vitro* detoxification enzyme inhibition assays identified a flavonoid compound, taxifolin, with high insect GST inhibitory activity from the four conifer species previously mentioned [19]. Besides taxifolin, numerous plant flavonoids, phenols and β-unsaturated carbonyl compounds are potent inhibitors of insect GSTs [24] indicating the potential for the discovery of new insecticide synergists that function through interference with conjugation-mediated detoxification in insects.

The objectives of the following research were to determine if the flavonoid, taxifolin, present in the four conifer N.A. species, would inhibit CPB detoxification enzymes *in vivo* and demonstrate activity as an insecticide synergist.

2. Materials and methods

2.1. Insect cultures

An insecticide-susceptible Colorado potato beetle strain (SS) has been maintained at Agriculture and Agri-Food Canada London, ON (AAFC) for more than 150 generations. A multiple insecticide-resistant Colorado potato beetle strain (RS) was obtained from the Department of Entomology, Michigan State University, East Lansing, MI. The strain was maintained there since 1997 (51 generations). Originally, it was collected in Long Island, NY and selected for imidacloprid-resistance once a year by exposing unmated 1–3 week old adult (both sexes) CPB to a dose that caused 90% mortality. The exposure was done by placing a 1 μl droplet of the imidacloprid LC_{90} dose (dissolved in acetone) onto the ventral abdomen of the adult insects. Surviving, pressured CPB were then reared on potato and the eggs collected to continue the imidacloprid-selected strain, the latest measurements indicated that it had increased in resistance by 5.1-fold since collection. The CPB sent to the AAFC London laboratory were maintained without exposure to insecticides for 7 generations over the course of the experiments. The CPB strains were reared on greenhouse grown potato *Solanum tuberosum* (Var. Kennebec) foliage and held at 25 ± 1 °C, 50 ± 5% RH and a photoperiod of 16:8 h (L:D). Plant growth and insect rearing methods followed those described in Wang et al. (2014).

2.2. Chemicals and plant extracts

Technical grade insecticides were obtained from commercial sources including, azinphos-methyl (>92% purity) (MANA, Norwalk, CT), carbofuran (99% purity) (Chem Service, West Chester, PA), and deltamethrin (99.4% purity) (Bayer Crop Science, Kansas, MO). The formulated insecticide, Guthion 50 WSB (50% azinphos-methyl) was supplied by MANA, Raleigh, NC, and pyrethrum extract was obtained from BASF, St. Louis MO. The enzyme inhibitors were purchased from commercial suppliers including, DEM and quercetin (Sigma-Aldrich, St. Louis, MO), DEF (Chem Service, West Chester, PA) and taxifolin (Extrasynthese, Genay, France). The 80% ethanol extracts of *P. banksiana* and *P. mariana* cones, and *A. balsamea* and *L. laricina* bark were prepared as described in Wang et al. (2014). Voucher specimens were deposited at the herbarium of the Biology Department, University of Ottawa, Canada.

2.3. Assessment of in vitro and in vivo inhibition activity

The assessment of enzyme inhibition and insecticide synergism was accomplished with 3 different ages of CPB larvae. An artificial diet bioassay with 1st instar larva and a topical exposure experiment with 2nd instar larva were both used since the diet assay allowed for a more accurate preparation of the synergist and insecticide concentrations, while the topical exposure could only be performed with larger sized CPB larvae and allowed for the screening of active combinations that could be further evaluated with the diet assay. The enzyme inhibition experiments were completed with 4th instar larvae since mid-gut and fat-body tissue could be more easily removed from the larger larvae.

2.3.1. In vitro esterase inhibition experiment

In vitro esterase assays were conducted using the substrate α-naphthyl acetate (α-NA) (Sigma-Aldrich) [25]. Reaction mixtures consisted of 50 μL CPB homogenate, which was extracted from RS CPB 4th instar larvae [19], and 2.5 μL of 25 mM α-NA, 2.5 μL enzyme inhibitors or conifer extracts at a range of concentrations and 195 μL of 0.02 M sodium phosphate buffer (pH 7.0). All assays were carried out in duplicate. Reaction mixtures were incubated for 10 min at 30 °C in a BioTek Synergy 2 plate reader. Reactions were stopped by adding 41 μL of Fast Blue B dye reagent (0.3% in 3.5% sodium dodecyl sulfate). Color was allowed to develop for 10 min before absorbance was measured against a blank cuvette containing the entire ingredient with inactivated enzyme. Absorbance was measured at 602 nm.

2.3.2. In vivo enzyme inhibition experiment

CPB 4th instar larvae were exposed to the enzyme inhibitors prior to sampling of insect tissues to measure the *in vivo* GSTs and total esterase activity. Sublethal concentrations of DEF (400 mg/L), DEM (6400 mg/L) and taxifolin (1024 mg/L) were dissolved in 100:1 water:ethanol and applied at 200 μL on both top and bottom of 4 cm diameter potato leaf disks. The leaf disks were dried for 20 min on a wire mesh and then transferred to a petri dish containing a Whatman #1 filter paper. Two 4th instar RS CPB larvae were placed on each leaf disk, and the petri dish sealed with a lid and held in an environmental chamber set at 25 ± 1 °C, 50 ± 5% RH and a photoperiod of 16:8 h (L:D). Each inhibitor treatment had six replicate disks and the trial was repeated three times. Control disks were treated with 1% ethanol and the CPB exposure followed the same procedure. Ten larvae from each treatment were dissected after 15 h and enzyme homogenates prepared [19].

In vivo GSTs activity was measured using the substrates 1-chloro-2, 4-dinitrobenzene (CDNB) and 1, 2-dichloro-4-nitrobenzene (DCNB) (Sigma-Aldrich, St. Louis, MO) in a 96 well microplate assay [26]. Briefly, each microplate well contained 300 μL of reaction mixture: 213 μL of 0.1 M sodium phosphate buffer (pH 6.5); 8 μL of acetone (solvent control) or 4.5 mg/mL DEM (positive control); 70 μL of enzyme incubated for 10 min at 30 °C; 6 μL of 50 mM CDNB or DCNB and 3 μL of 100 mM reduced glutathione (GSH) (Sigma-Aldrich) added to start the reaction. The change in absorbance at 340 nm was recorded for 6 min with a BioTek Synergy 2 plate reader (Winooski, VT). Wells containing 70 μL of boiled enzyme were used as a negative control. *In vivo* esterase activity was measured using two substrates, α-NA and β-naphthyl acetate (β-NA), and followed the same procedure as the *in vitro* esterase assay except that the enzyme inhibitors were removed from the reaction system. The concentration of α-naphthol and β-naphthol (Sigma-Aldrich)

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