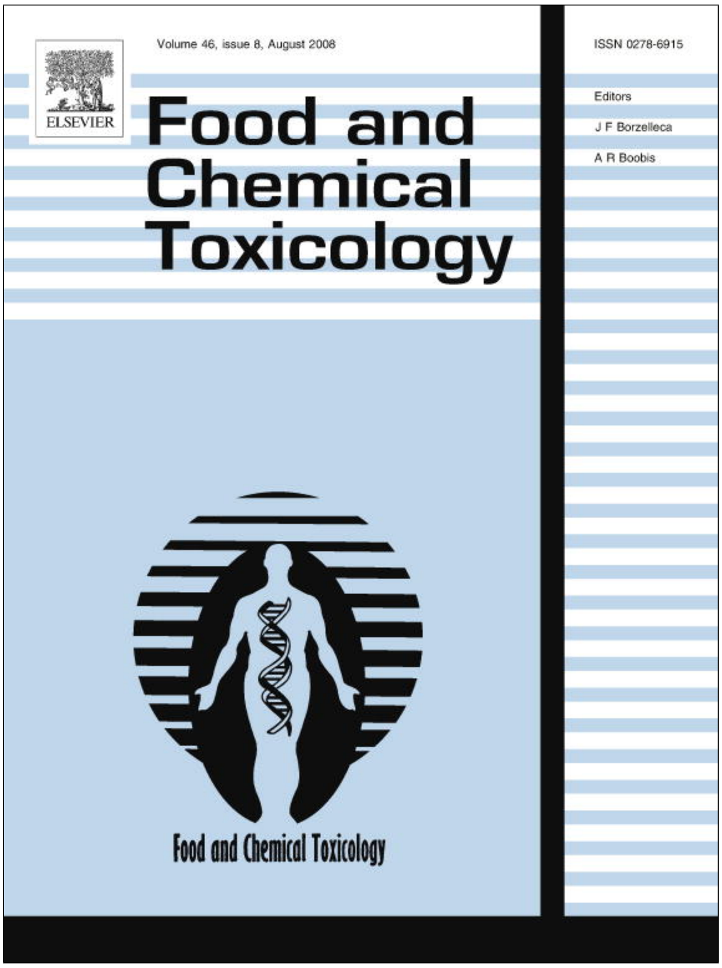
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Glycoalkaloid responses of potato to Colorado potato beetle defoliation

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ABSTRACT

Two experiments were conducted to measure the glycoalkaloid concentrations of potato tubers in response to Colorado potato beetle and manual defoliation. For plants defoliated by Colorado potato beetles, there was a significantly greater production of glycoalkaloids than in control plants and manually defoliated plants for both skin and inner tissue of tubers in experiment 1. In experiment 1, there was a 58.1% and 48.3% increase in glycoalkaloids in skin and inner tissue of tubers, respectively, from plants defoliated at high levels by Colorado potato beetles compared to control plants. In experiment 2, although a significant difference in glycoalkaloid concentration was not observed among the treatments, the skin and inner tissue of tubers from plants defoliated at high levels by Colorado potato beetles increased glycoalkaloid concentration by 23.4% and 14.5%, respectively, compared to tubers from control plants. In experiment 1, the concentration of tuber extract required to reduce Chinese hamster ovary (CHO) cellular proliferation by 50% was 10-fold less for the skin versus the inner tissue, indicating that skin tissue was more toxic under the in vitro conditions of this assay.

1. Introduction

Many plant secondary compounds serve as natural pesticides, and there is increasing interest to enhance these natural pesticides for commercial use (Fenwick et al., 1990). Plants are being bred to contain not only a greater diversity of natural compounds, but also greater quantities (Hlywka et al., 1994). However, at certain concentrations, these compounds can be toxic to humans and other animals (Theis and Lerdau, 2003).

Although terpenes are the largest class of secondary metabolites (Theis and Lerdau, 2003), glycoalkaloids are thought to be the most highly consumed natural toxin in North America (Hall, 1992). Little is known about the human dietary risks associated with consumption of these chemicals or how the dietary risks change in response to insect herbivory.

In potato (*Solanum tuberosum* L.), glycoalkaloids function as natural defense mechanisms against pathogens and insects (Lachman et al., 2001). Because naturally occurring pesticides often are synthesized when plants are under stress, it is expected that injury to plant tissue would instigate synthesis of higher concentrations of these compounds in the injured versus uninjured plant tissue. Hlywka et al. (1994) found that tubers from plants subjected to Colorado potato beetle (*Leptinotarsa decemlineata* Say) defoliation contained higher glycoalkaloid concentrations than tubers from undefoliated plants.

In the potato plant, glycoalkaloids are found in high concentrations in the leaves, stems, and sprouts. Relatively lower concentrations of glycoalkaloids can be found in the skin of tubers and areas where sprouts emerge (Lachman et al., 2001). Friedman and Dao (1992) found that leaves had a concentration of glycoalkaloids 10 times greater than the tubers and a sprout glycoalkaloid concentration nearly 68 times greater than the tubers. Phillips et al. (1996) observed a greater concentration of glycoalkaloids in the leaves compared to tubers from the same plants; however, there was a great deal of variability among leaf glycoalkaloid concentrations within the same variety of plants. In tubers, the greatest concentration of glycoalkaloids was found in the skin (Bejarano et al., 2000), and the greater the concentration of glycoalkaloids present in tubers, the more bitter the taste (Lachman et al., 2001).

Although there are many glycoalkaloids, α-chaconine and α-solanine make up 95% of the total glycoalkaloids present (Friedman and McDonald, 1997); α-solanine is found in greater concentrations than α-chaconine, and α-solanine has only half as much specific toxic activity as α -chaconine (Lachman et al., 2001). Other glycoalkaloids that are present, but in much lower concentration, are β- and γ-solanines and chaconines, α- and β-solamarines, aglycones demissidine, and 5-β-solanidan-3-a-ol (Friedman and McDonald, 1997).

Abbreviations: ANOVA, analysis of variance; CHO, Chinese hamster ovary; DW, dry weight; ELSD, evaporative light scanning detector; FW, fresh weight; HPLC, high performance liquid chromatography; RCBD, randomized complete block design; RSD, relative standard deviation; USDA, US department of agriculture.

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Factors that increase glycoalkaloid levels in tubers include tuber exposure to light, bruising, cutting, rotting by fungi or bacteria, and other forms of mechanical damage (Lachman et al., 2001). Lachman et al. (2001) found that damaged tubers had 89% higher glycoalkaloid content than undamaged tubers. In 1994, when weather conditions were unfavorable and dry, the glycoalkaloid content was 71% higher than the content in 1995. In a greenhouse study, tubers harvested from a "hot" versus a "cool" chamber contained a greater concentration of glycoalkaloids (Lachman et al., 2001). In addition to growing conditions, handling, and storage, tubers exposed to light often turn green and can have especially high glycoalkaloid concentrations (Friedman and McDonald, 1997).

The US department of agriculture (USDA) has recommended a food-safety level for glycoalkaloids of 200 mg/kg fresh weight (FW) or 1000 mg/kg dry weight (DW) (Bejarano et al., 2000; Zeiger, 1998). However, neither α-solanine nor α-chaconine are regulated in the US. Most commercial tablestock tubers contain between 20 and 130 mg/kg FW (Zeiger, 1998) or 133 and 867 mg/kg DW.

Using the early-maturing potato cultivar 'Superior' in field experiments, Hlywka et al. (1994) showed that Colorado potato beetle injury of leaves increased glycoalkaloid concentrations in tubers. However, they did not examine toxicological responses or assess potential human dietary risks. The objectives of this study were to determine the glycoalkaloid content of 'Russet Burbank' potatoes, following Colorado potato beetle and manual defoliation in greenhouse experiments. Responses were measured by determining the concentration of glycoalkaloid production and the percentage of tuber extract that caused a 50% inhibition in mammalian cellular proliferation for the skin and innermost flesh of tubers at different levels of Colorado potato beetle and manual defoliation. In Pariera Dinkins and Peterson (2008), we estimate the potential human dietary risks associated with consumption of potatoes in the presence and absence of Colorado potato beetle injury.

2. Materials and methods

2.1. Plant material

All plants were grown in a greenhouse (Montana State University, Bozeman, MT) and planted in 50:50 MSU:Sunshine #1 soil mix, and fertilized weekly with Scott's Peter Professional Peat-Lite Special 20-20-20. The Sunshine Mix #1 consisted of Canadian Sphagnum Peat Moss, perlite, vermiculite, starter nutrient charge, wetting agent, and Dolomitic lime (Sun Gro Horticulture, Inc., Bellevue, WA). The MSU soil mix consisted of equal parts of Bozeman Silt Loam Soil, washed concrete sand, and Canadian Sphagnum Peat Moss in addition to AquaGro 2000 G wetting agent blended in at 593 g/m^3 of soil mix.

Plants were grown at 21 ± 2 °C with a photoperiod of 14:10 (Light:Dark). The cultivar, Russet Burbank, was obtained from VenHuizen Seed Potatoes, Inc., Belgrade, MT. Each seed tuber was cut, ensuring at least one eye per cut, and placed cut-side down in a 20-L pot filled approximately with 12–13 cm of pre-moistened 50:50 MSU:Sunshine soil mix and covered 5–9 cm with pre-moistened 50:50 MSU:Sunshine soil mix.

Plants were watered 4–5 times weekly and fertilized with Scott's Peter Professional Peat-Lite Special 20-20-20 bi-weekly. Once plants reached approximately 9-cm tall, in a process called "hilling," soil was added to the pots weekly until pots were full.

In a greenhouse, the experiment was arranged within a randomized complete block design (RCBD). Metal halide lamps (1000 W) served as the blocking factors. The following defoliation treatments were used: control (no defoliation), low, medium, and high Colorado potato beetle and manual defoliation. The treatment factors were replicated five times and the experiment was replicated twice (2004 and 2005).

At the early vegetative stage, all plants were individually covered with nets approximately 91 cm × 40 cm × 40 cm ($h \times w \times d$) made of white tulle and Colorado potato beetle eggs were obtained from the Phillip Alampi Beneficial Insect Lab, New Jersey Department of Agriculture, Trenton, NJ. Approximately 120 egg masses were placed on approximately 10–15 extra potato plants and allowed to hatch and feed until approximately the third instar. Just before flowering, approximately 15, 20, and 25 third instars were applied to the low, medium, and high treatment plants and allowed to defoliate. Third and later instars were used because feeding by first and second instars make up less than 10% of total consumption and have relatively high levels of mortality. Once the low, medium, and high defoliation plant leaf area was reduced by approximately 30% (low), 60% (medium), and 90% (high), respectively, the Colorado potato beetles were removed. The manually defoliated plants were defoliated with scissors weekly to simulate the percentage and patterns of leaf mass removed by the Colorado potato beetles.

Upon senescence, stems were cut and potatoes were harvested two weeks later. The tubers were washed, placed in brown paper bags, and the bags were stored in a dark cold room held at 4 °C. All tubers from each experimental unit were placed in separate bags.

2.2. Glycoalkaloid analysis

Stored Russet Burbank tubers of approximately the same size across treatments were separated into three tissue samples: the skin, the outer flesh, and the inner core. Tubers were skinned approximately 2 mm from the surface using a vegetable peeler and approximately 50% of the remaining tissue was separated to consist of 50% of outer flesh and the other half the inner core. These three tissue samples were cut into smaller pieces, dipped into liquid nitrogen, placed into 50-ml centrifuge tubes with a cotton cap, immediately placed in a 1200-ml fast freeze flask and freeze dried for approximately 60 h using a freeze dryer (LABCONCO Freeze Dry System/Freezone 4.5, Labconco Corporation, Kansas City, MO).

After 60 h, the samples were removed and ground using a Black & Decker HandyChopper Plus™ (Model HC3000, Towson, MD) until the consistency of a fine powder was achieved. The powder was placed into 50-ml centrifuge tubes and stored in a −60 °C freezer.

Once all samples were freeze dried and ground, the control (no defoliation), high manual defoliation, and high Colorado potato beetle defoliation treatments (24 total samples), were coded and sent to Eurofins Scientific (Petaluma, CA) for glycoalkaloid quantification using high performance liquid chromatography (HPLC). Because of cost considerations, only samples from inner and skin locations were analyzed.

The diluent was prepared by combining water, acetonitrile, and 85% phosphoric acid (80:20:0.1 (v/v/v)). In a 25-ml volumetric flask, the standard stock solution was prepared by using approximately 3.5 mg of α-chaconine and 4.5 mg of α-solanine diluted to volume using the diluent. Standard 1 was prepared by using 2 ml of the stock standard solution diluted to volume using the diluent in a 100-ml volumetric flask. Standard 2 was prepared by combining 2 ml of the standard stock solution in a 50-ml volumetric flask diluted to volume using the diluent. In a 50-ml flask, standard 3 was prepared by diluting 3 ml of the standard stock solution to volume using the diluent. Standard 4 was prepared using a 25-ml volumetric flask with 2 ml of the stock standard solution diluted to volume using the diluent. Standard 5 was prepared by diluting 2 ml of the standard stock solution to volume using the diluent in a 10-ml volumetric flask.

The stock standard solution and each standard solution were injected twice prior to actual sample injections, after every 16th sample injection, and upon completion of all sample injections. The standard curve was created using exponential curve fitting and the y-intercept, correlation coefficient, and percent relative standard deviation (RSD) of the standard curves for α-solanine and α-chaconine were calculated. The concentration of α-solanine and α-chaconine per sample were quantified using the standard calibration curves. High RSDs of the standard curves are typical for evaporative light scanning detector (ELSD) detection when calculated more than two orders of magnitude in concentration.

Approximately 500 mg of sample was weighed, transferred to a 15-ml centrifuge tube, combined with 10 ml of diluent, and shaken for approximately 10 min on a wristaction shaker. The tube was then sonicated for 15 min, allowed to cool to room temperature, centrifuged for 10 min, and the supernatant was filtered through a 0.45-μm PTFE filter.

Each sample was run twice through a Dionex Summit HPLC with ELSD and Dionex Chromeleon software. The Dionex Summit HPLC was fitted with an Alltech Altima HP C-18 Amide (150-mm × 4.6-mm, 5-μm) column at 25 °C. The mobile phase consisted of A – 0.1% trifluoroacetic acid in water and B – acetonitrile with a pump program of 10% B to 34% B over a 36-min period. The flow rate was set at 1.0 ml/min with an injection volume of 50 μL. The detection was ELSD. The drift tube temp was set at 110 °C, the gas flow was set at 3.0 L/min, and the impactor was off.

2.3. Cellular proliferation analysis

The protocol was based on Sayer et al. (2006) and evaluated the ability of extracts found in the skin and inner tissue of potatoes from plants with varying levels of Colorado potato beetle defoliation to inhibit Chinese hamster ovary (CHO-K1-BH4) cell proliferation. Inhibition of cell proliferation by the potato samples was evaluated by comparing the number of cells in each treatment to the untreated control.

Once all potato samples were freeze dried and ground, four replicates of the control (no defoliation), high manual defoliation, and high Colorado potato beetle defoliation treatments (24 total samples) were coded and sent to J.E. Gibson (East Carolina University, Greenville, NC) in 2005 for a blind analysis.

CHO-K1-BH4 cells were obtained from G.D. Charles (Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI). CHO-K1-BH4 cells were grown in 25-cm^2 plastic culture flasks containing 5 ml of F-12 nutrient media (Ham) (Invitrogen, Carlsbad, CA) combined with 5% fetal bovine serum (Hyclon, Logan, UT), 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 2.5 µg/ml of Fungizone Amphotericin B (Invitrogen, Carlsbad, CA). The cells were seeded at a density of 1 × 105 cells/ml for 24 h before treatment. Then the culture medium was replaced with a new medium containing the tested extract with different concentrations and incubated at 37 °C for 48 h. The medium without extract was used as the untreated control and ethanol with different concentrations correspondingly served as positive control.

Dry potato samples from the control, high manual defoliated, and high Colorado potato beetle defoliated treatments were extracted in the F-12 nutrient media (Ham) at a ratio of 0.05-g sample/ml of nutrient media for 24 h, followed by centrifugation and sterilization of the supernatant using a 0.2-µm Whatman sterile syringe filter, CA filter media fitted with polypropylene hosing. The supernatant for each treatment (control, high manual defoliation, and high Colorado potato beetle defoliation) was diluted to 0%, 0.4%, 0.8%, 1.2%, 1.6%, 2%, 4%, 6%, 8%, and 10% using the nutrient media. The positive control consisted of 0%, 1%, 2%, 3%, 4%, and 5% ethanol.

In 6-well plates, each well consisted of 2 ml of culture media combined with each dilution percentage and 100,000 CHO-K1-BH4 cells. The plates were returned to the incubator for 48 h under the same conditions as above. Two plates (subsamples) per dilution were used.

After 48 h in the incubator, the plates were removed, the media was aspirated, and the cells were trypsinized and re-suspended in 1 ml of the culture media. Each well was counted for CHO-K1-BH4 cells using a coulter counter (Z2 Duel Threshold Coulter Counter, Beckman Coulter, Fullerton, CA). Treatment cell counts were compared to the untreated control cell counts from the wells on the same plate. We assumed that 100% of the cells from the untreated control were considered capable of proliferating. The inhibitory concentration (IC_{50})—the dilution necessary to cause a 50% reduction in cellular proliferation—was determined by using a probit regression.

2.4. Statistical analyses

Control and treated groups were compared ($\alpha = 0.05$) using analysis of variance (ANOVA) and treatment means were separated by TukeyHSD multiple comparison of means using 95% family-wise confidence levels. Data were analyzed using R 2.0.1 (R: A Language and Environment for Statistical Computing; R Development Core Team, The R Foundation for Statistical Computing, 2004, Vienna, Austria).

3. Results

3.1. Glycoalkaloids

For experiment 1, treatment ($F = 6.8$; $df = 2$; $P = 0.008$), tissue type ($F = 271.62$; $df = 1$; $P < 0.0001$), and the interaction between treatment and tissue type ($F = 4.67$; $df = 2$; $P = 0.027$) all had a significant effect on glycoalkaloid concentrations. Tuber glycoalkaloid concentrations from plants with high Colorado potato beetle defoliation had a significantly greater glycoalkaloid concentration (2442.5 [skin] mg/kg DW and 258 [inner] mg/kg DW) than control plants (1545 [skin] mg/kg DW and 174.5 [inner] mg/kg DW). Glycoalkaloid concentrations were significantly greater in the skin versus the inner tissue (Figs. 1 and 2).

Unlike experiment 1, tissue type ($F = 88.72$, $df = 1$, $P < 0.00001$) was the only factor to have a significant effect on glycoalkaloid concentrations in experiment 2. Glycoalkaloid concentrations in the skin were significantly greater than in the inner tissue. Glycoalkaloid concentrations between treatments were not significantly different (Figs. 1 and 2).

For experiment 1, the skin glycoalkaloid concentrations from control, high manually defoliated, and high Colorado potato beetle defoliated plants were 1545 mg/kg DW, 1987.5 mg/kg DW, and 2442.5 mg/kg DW, respectively (Fig. 1). For experiment 2, the skin glycoalkaloid concentrations from control, high manual defoliated, high Colorado potato beetle defoliated plants were 1349.8 mg/kg DW, 1695.5 mg/kg DW, and 1665 mg/kg DW, respectively (Fig. 1). For experiment 1, the inner tissue glycoalkaloid concentrations from control, high manual defoliated, high Colorado potato beetle defoliated plants were 174.5 mg/kg DW, 149.4 mg/kg DW, and 258.8 mg/kg DW, respectively (Fig. 2). For experiment 2, the inner tissue glycoalkaloid concentrations from control, high manual defoliated, and high Colorado potato beetle defoliated plants were 404.9 mg/kg DW, 383.6 mg/kg DW, and 463.8 mg/kg DW, respectively (Fig. 2).

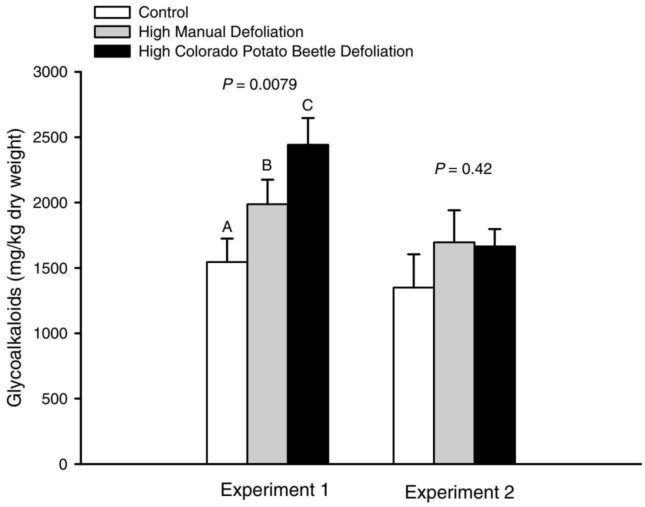

Fig. 1. Glycoalkaloid concentrations for the skin of potatoes harvested from control plants (0% defoliation), high manual defoliation (~90% defoliation), and high Colorado potato beetle defoliation (~90% defoliation) for experiments 1 and 2. Bars followed by different letters are significantly different, $\alpha = 0.05$).

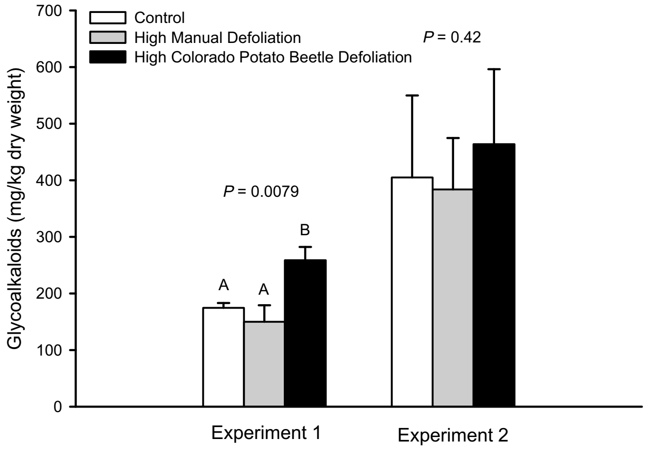

Fig. 2. Glycoalkaloid concentrations for the inner tissue of potatoes harvested from control plants (0% defoliation), high manual defoliation (~90% defoliation), and high Colorado potato beetle defoliation (~90% defoliation) for experiments 1 and 2. Bars followed by different letters are significantly different, $\alpha = 0.05$).

3.2. In vitro toxicity

Inhibitory concentration (IC_{50}) was the concentration of potato extract that caused 50% reduction in proliferation of CHO cells. Because only experiment 1 was observed to have a significant treatment effect, only experiment 1 was used to evaluate the IC_{50} values. Tissue type ($F = 25.786$; $df = 1$; $P = 0.0004$) was the only factor to have a significant effect on IC_{50}; the inner tissue required a significantly greater amount of extract to elicit a 50% reduction in cellular proliferation (12.523 µg/ml) than the skin (1.411 µg/ml) (Fig. 3).

4. Discussion

For plants defoliated by Colorado potato beetles, there was a significantly greater production of glycoalkaloids than in control

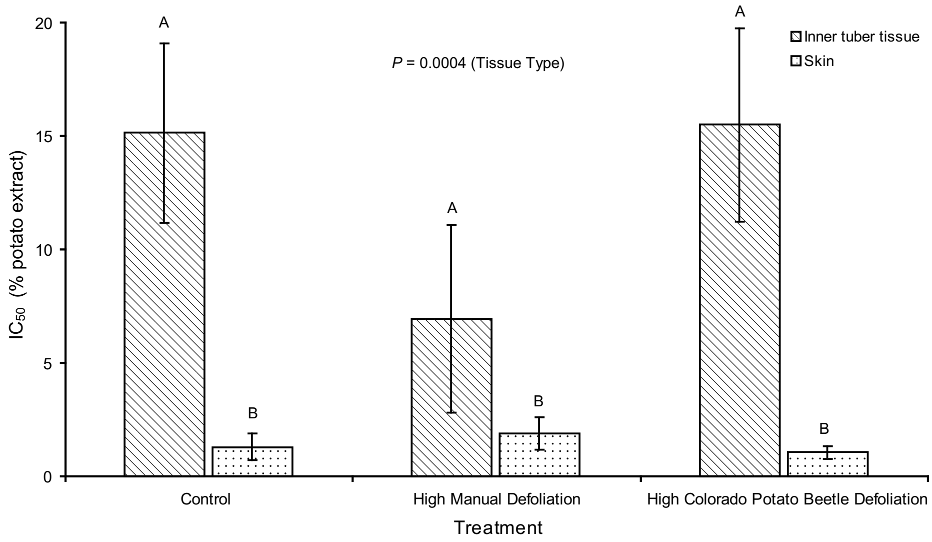

Fig. 3. Mean IC_{50} values (inhibitory concentration, 50%) from potato tuber extracts of control, high manual defoliated, and high Colorado potato beetle defoliated plants from experiment 1. Bars followed by different letters are significantly different, $\alpha = 0.05$).

plants and manually defoliated plants for both skin and inner tissue in experiment 1. In field experiments, Hlywka et al. (1994) observed a 37.5% increase in glycoalkaloid concentration of tubers from 'Superior' plants defoliated at high levels by Colorado potato beetles in comparison to control plants. However, there were no significant increases associated with manual defoliation. In the greenhouse experiments presented here, using a different cultivar, 'Russet Burbank', similar results were observed. In experiment 1, there was a 58.1% and a 48.3% increase in skin and inner tissue glycoalkaloid concentration of tubers, respectively, from plants defoliated at high levels by Colorado potato beetles compared to control plants. In experiment 2, although a significant difference in glycoalkaloid concentration was not observed among the treatments, the concentration of glycoalkaloids in the skin and inner tissue of tubers from plants defoliated at high levels by Colorado potato beetles were greater by 23.4% and 14.5%, respectively, compared to tubers from control plants. The lack of significance in experiment 2 may be a result of differences in storage length; experiment 1 tubers were stored for a greater period of time than experiment 2. Because significant differences were observed in experiment 1 and a similar trend was observed in experiment 2, increases in tuber glycoalkaloids, overall, seem to be a general response of potato to Colorado potato beetle defoliation.

Hlywka et al. (1994) observed that manually defoliated plants and injury by potato leafhoppers (*Empoasca fabae* Harris) did not elicit increases in glycoalkaloid production in tubers whereas tubers from plants injured by Colorado potato beetles had increases in tuber glycoalkaloid concentrations. Because of the similar response in glycoalkaloid production observed between control plants, manually defoliated plants, and plants injured by potato leafhoppers, it is illustrative of a specific interaction occurring between the potato plant and the Colorado potato beetle; this response may be either specific to Colorado potato beetles or specific to the type of injury, i.e., defoliation, that Colorado potato beetles impose.

Although the mechanism for the initiation of glycoalkaloid synthesis has yet to be determined, glycoalkaloids cannot be translocated within plants (Roddick, 1982) and any increases observed in the tuber as a result of injury to the plant have to be elicited by a signal from the plant and received by the tuber. Therefore, a signal-response mechanism most likely exists. In the case of the Colorado potato beetle, because injury is occurring strictly to the leaves, it seems that it is a plant signal-response and as a result, the tuber initiates increased synthesis of glycoalkaloids. The Colorado potato beetle may initiate the signal either through interactions between plant cells and saliva or because Colorado potato beetles defoliate leaves by lysing cells little by little.

Hlywka et al. (1994) hypothesized that the application of various phytohormones to plant foliage and/or directly on the tuber itself may elicit increases in glycoalkaloid concentrations. Alternatively, they hypothesized that because action potentials typically occur during plant injury and stress and are capable of "long-distance signal transduction from foliage to tubers", they too may elicit increases in glycoalkaloid concentrations. The systemic expression of a proteinase inhibitor gene via a phytohormone signal involving abscisic acid can result from defoliation of plant leaf mass (Peña-Cortés et al., 1989). Bergenstråhle et al. (1992) did not observe that abscisic acid added to tuber disks affected the induction of increased levels of glycoalkaloids. Because of this, Hlywka et al. (1994) suggested that action potentials most likely were responsible for producing increases in glycoalkaloids in potatoes. Pariera Dinkins (2006) did not observe effects of Colorado potato beetle defoliation on photosynthetic rates of potato leaves, even though glycoalkaloid concentrations were higher for tubers on injured plants. This may indicate that glycoalkaloid production is not energetically expensive.

The concentration of tuber extract required to reduce CHO cellular proliferation by 50% was 10-fold less for the skin versus the inner tissue, indicating that skin tissue is much more toxic under the in vitro conditions of this assay. This most likely is because tuber skin contains greater concentrations of glycoalkaloids than inner tissue. However, glycoalkaloids were not quantified from the extracts used in this study.

Despite a different potato cultivar and greenhouse versus field conditions, our results were consistent with Hlywka et al. (1994). Therefore, increasing glycoalkaloid concentrations seem to be a general response of potato to Colorado potato beetle defoliation. The risks to humans posed by increases in glycoalkaloids in tubers can be estimated with dietary exposure and risk methodologies (Pariera Dinkins and Peterson, 2008). Because plants are being bred to contain not only a greater diversity of natural compounds, but also greater quantities (Hlywka et al., 1994), breeders should be aware that if a plant is bred to have higher baseline glycoalkaloid levels, tubers could contain unacceptable levels after Colorado potato beetle infestations or other forms of mechanical damage—and possibly unacceptable levels even in the absence of injury (Pariera Dinkins and Peterson, 2008).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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