American Journal of Potato Research
https://doi.org/10.1007/s12230-024-09963-5

Rapid Screening of Colorado Potato Beetle Resistance Derived from *Solanum okadae*

Hanna J. McCoy[1,2] · Sean Fenstemaker[3] · Pamela MacKinley[1] · Jess Vickruck[1] · John Bamberg[3] · Larry A. Calhoun[2] · Helen H. Tai[1]

Accepted: 30 July 2024

Abstract
Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) is a major insect pest of potato and development of resistant varieties is part of a strategy for management. Wild relatives of potato are resources for genetic improvement through breeding. Interspecies crosses to introgress CPB resistance will be facilitated with rapid and inexpensive selection methods. *Solanum okadae* is a novel source of feeding deterrence against the beetle and was associated with production of a naturally occurring leaf-specific lactone-containing metabolite in the foliage. The Baljet assay has been used for decades in pharmaceuticals for rapid screening of lactone-containing compounds. A modified Baljet assay was developed for potato foliar tissue to rapidly screen for lactone-containing compounds in plants from the field, greenhouse, and laboratory. Herein we report the screening of potato foliage for CPB resistance with a Baljet assay validated by CPB larval feeding studies. Foliage from wild accessions of *S. okadae* were tested using the Baljet assay and results showed that production of the leaf-specific lactones has a large range of variation in the species. In addition, tubers tested using the Baljet assay had negligible levels of lactone-containing metabolites, confirming leaf-specific production of lactones. This inexpensive method using leaf disk screening will allow potato breeders to quickly select for potential CPB resistant germplasms and advance the breeding of sustainable crops.

Keywords Baljet assay · Potato · Cardiac glycoside · Plant host resistance to herbivores · Colorado potato beetle · Crop wild relatives

Abbreviations

CPB	Colorado potato beetle
OKA15	*Solanum okadae*
SARS-CoV-2	Severe acute respiratory virus 2
NaOH	Sodium hydroxide
LC_{90}	Lethal concentration 90

Introduction

Colorado potato beetle (CPB) is a major herbivorous pest of potato, with infestations causing defoliation associated with yield losses (Alyokhin et al. 2022). Plants have evolved defense mechanisms against herbivory, including production of secondary metabolites in foliage (Bennett and Wallsgrove 1994; Erb and Kliebenstein 2020). The potato plant in particular produces the secondary metabolites solanine and chaconine, which are glycoalkaloids toxic to mammals but ineffective in defense against CPB (Wierenga and Hollingworth 1992; Friedman 2006; Tai et al. 2014). CPB are known to readily adapt to control measures (Cingel et al. 2016), and implementing different approaches to manage the pest are vital. Breeding for host resistance in potato is a strategy used to prevent CPB feeding (Tai and Vickruck 2022). Intercrossing CPB resistant wild relatives with cultivated potato to improve breeding germplasm is a way to move towards a sustainable production system with reduced

✉ Helen H. Tai
Helen.Tai@agr.gc.ca

[1] Agriculture and Agri-Food Canada Fredericton Research and Development Centre, 95 Innovation Road, P.O. Box 20280, Fredericton, NB E3B 4Z7, Canada

[2] Department of Chemistry, University of New Brunswick, P.O. Box 4400, Fredericton, NB E3B 5A3, Canada

[3] USDA/Agricultural Research Service, Potato Genebank, 4312 Hwy 42,, Sturgeon Bay, WI 54235, USA

Published online: 14 August 2024

need for insecticides while improving yields (Tai and Vickruck 2022). In a screening of potato wild relatives for field resistance to CPB, *S. okadae* was identified for its host resistance (Pelletier et al. 2001).

Host resistance in wild relatives of potato is often associated with compounds produced in the foliage of plants (Tai and Vickruck 2022). Identification of the compounds involved in CPB interaction in wild species is critical to understanding the mechanism of resistance and developing breeding strategies (Pelletier et al. 2001). Previous studies have identified that leptine and dehydrocommersonine glycoalkaloids are associated with CPB resistance in *S. chacoense*, *S. brevicaule* (*oplocense*) and *S. commersonii* (Tai et al. 2014, 2015; Sinden et al. 1986; Wolters et al. 2023; Paudel et al. 2019; Kaiser et al. 2020; Yencho et al. 2000). Accordingly, additional wild relatives of potato with resistance to CPB were screened using a non-targeted metabolomics approach and found to produce different glycoalkaloids as well as other classes of foliar metabolites such as flavonoid glycosides and coumarins (Tai et al. 2014). A CPB resistant clone of the wild species *S. okadae*, named OKA15, produced high levels of a possible cardiac glycoside – a class of lactone-containing bioactive secondary metabolites associated with insect deterrence – in the foliage (McCoy et al. 2022). The foliar production of cardiac glycosides would be a new mechanism of CPB resistance for potato (McCoy et al. 2022).

Cardiac glycosides (Fig. 1) are steroidal molecules produced in plants and animals as defence mechanisms (Bejček et al. 2021). This class of compounds has a lactone ring attached to a sterol derived from cholesterol biosynthesis (Bejček et al. 2021). There are two subclasses of cardiac glycosides that differ by the type of unsaturated lactone ring present (Bejček et al. 2021). Cardenolides are commonly found in plants, such as milkweed and *Digitalis* species, and contain a 5-membered furanone ring (Yamane et al. 2010; Bejček et al. 2021). Bufadienolides contain a 6-membered pyranone ring and were first isolated from toad (*bufo*) venom (Mukherjee 2019; Bejček et al. 2021). Traditionally, cardiac glycosides were isolated from *Digitalis* species – foxglove – for medicinal applications (Norn and Kruse 2004; Akinmoladun et al. 2014). Historically these compounds were used to coat arrows for hunting (Haviv and Karlish 2013). More recently, the biological activity of cardiac glycosides has been employed in pharmaceuticals for the compound's ability to alter cardiac activity (Yamane et al. 2010). The effect of cardiac glycosides, such as ouabain, on the Na^+/K^+-ATPase is known to affect cardiac muscle contractions, resulting in effective treatment of cardiac arrhythmias and heart failure (Yamane et al. 2010). The size and complexity of these molecules creates challenges for laboratory synthesis (Ainembabazi et al. 2022). Cardiac glycosides are continuously sourced from plant material and extensively researched for new applications, including cancer and SARS-CoV-2 treatment (Bejček et al. 2021; Souza et al. 2021).

The Baljet assay has been used for decades in the pharmaceutical industry to test drug components for the presence of cardiac glycosides (Neuwald 1950). In plants, traditionally the assay was applied to screen leaves of *Digitalis* species for these cardiac glycosides to be further processed for medicinal practices (Neuwald 1950). The Baljet assay is a colorimetric assay that uses a sodium picrate solution to indicate the presence of cardiac glycosides upon a colour change from yellow to orange (Neuwald 1950). This bathochromic shift is a result of the reaction between the unsaturated lactone ring of the cardiac glycoside and the poly-nitroaromatic ion in alkaline medium (Fig. 2) (Morsy 2017). The resulting Meisenheimer complex has a characteristic absorbance around 490 nm (Korchagina and Petrova 1979). Although the assay is known to have a lack of specificity, it is widely practiced as an effective initial screening method for the detection of cardiac glycosides (Morsy 2017). The Baljet assay was applied in the current study to assess for possible cardiac glycosides in potato tissues. Herein, we report on the development of a high throughput and inexpensive technique to screen for the lactone-containing compounds derived from *S. okadae* clone OKA15 in potato foliage and tubers. Validation of the role of putative foliar cardiac glycosides in host plant resistance was done

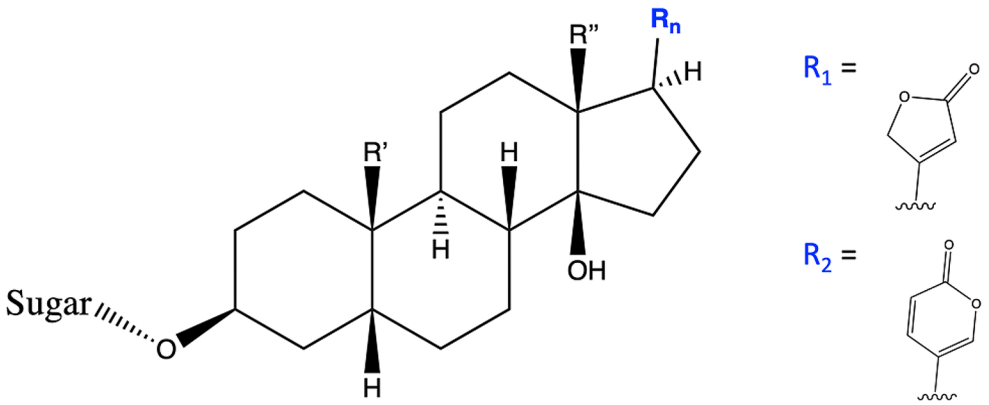

Fig. 1 General structure of a cardiac glycoside. There are two subclasses of cardiac glycosides depending on the type of lactone ring: cardenolides (R_1) and bufadienolides (R_2)

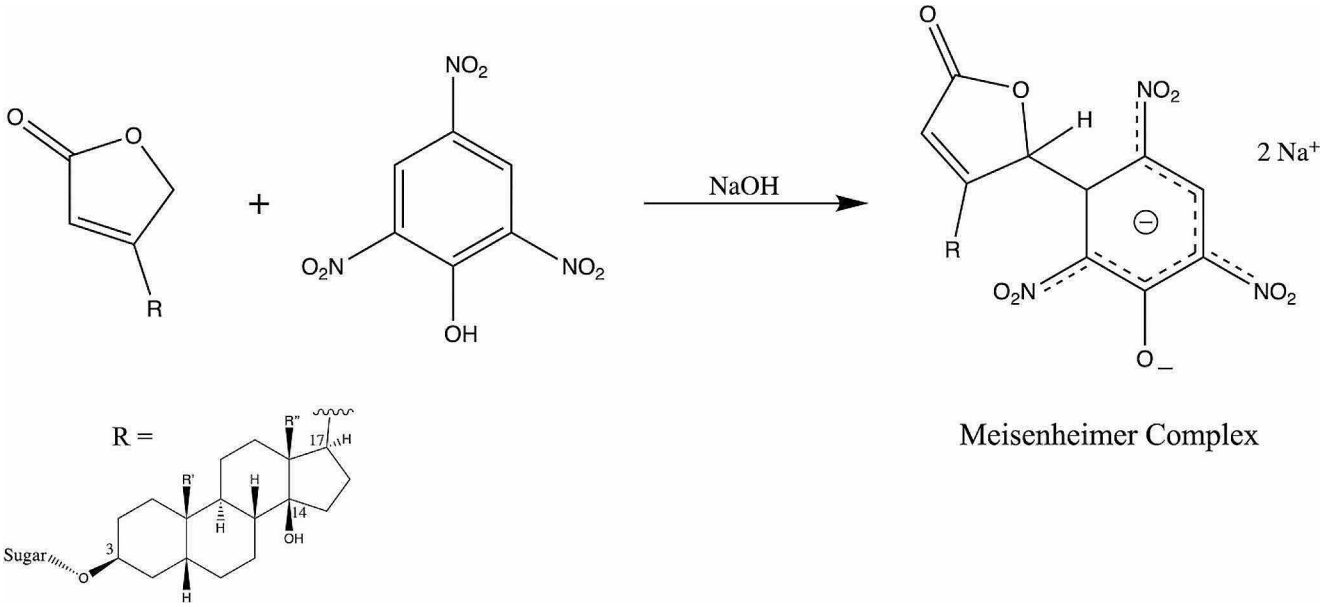

Fig. 2 General reaction mechanism of poly-nitroaromatic picric acid with the unsaturated lactone ring of cardiac glycosides in alkaline medium to produce the corresponding Meisenheimer complex

using CPB feeding assays. Moreover, the range of variation in foliar levels of lactones in a natural population of *S. okadae* held at US Potato Genebank was assessed using the improved Baljet assay to demonstrate its application in screening field-grown plants.

Materials and Methods

Statistical Analysis Software

All statistical analyses reported herein were performed in RStudio 2023.3.0.386 running R version 4.2.3 (R Core Team 2023) using the *readxl* (Wickham and Bryan 2023), *dplyr* (Wickham et al. 2023), *ggplot2* (Wickham 2016), *RcolorBrewer* (Neuwirth 2022), *ggpubr* (Kassambara 2023), and *car* (Fox and Weisberg 2019) packages.

Propagation and Collection of Foliar Tissue

OKA15 and Kennebec Greenhouse Propagation

Wild *S. okadae* plants were obtained as true botanical seeds from the United States Potato Genebank (accession number PI 458367). The seeds were sown on MS agar (4.4 g of Murashige and Skoog basal salts with vitamins, 30 g of sucrose, and 7 g/L plant tissue culture agar), germinated, and transferred to the greenhouse as previously described (McCoy et al. 2022). A unique seedling – named OKA15 – was selected from the population for further analysis due to its compatibility with tissue culture propagation. The domesticated *S. tuberosum* cv. Kennebec was propagated from tuber seed sourced from the Benton-Ridge Substation of Fredericton Research and Development Centre, Agriculture and Agri-Food Canada (AAFC), Benton, New Brunswick, Canada. The OKA15 and Kennebec plants were left to grow in the greenhouse in 6-in. pots with potting mix at the Fredericton Research and Development Centre, AAFC. A weekly fertilizer application of 20-20-20 was applied to both species. Fresh leaflets were collected from the middle section of fully grown OKA15 and Kennebec plants for use in the reported CPB feeding studies and Baljet assays.

Field Trial

The material used in this study originated from adapted Phureja clones, sourced from Dr. Kathleen Haynes (USDA/ARS) (Haynes et al. 2019), which were bulk intermated. Three clones from this seed population were used in further crosses. These three adapted Phureja clones were used in bulk intermating with six clones derived from *S. okadae* PI 458,367 seedlings. Progeny from this cross underwent selection for long-day adaptation and beta carotene accumulation in tuber flesh. Twenty of the resulting F1 seedlings from this cross were grown out and used in bulk intermating to generate the F2 population. Intermated F_2 progenies (128 individuals) derived from *S. okadae* (PI 458367) and a *S. tuberosum* Group Phureja (adapted Phureja) were randomly planted as single plant plots with 0.5 m within row spacing at the Hancock Agricultural Research Station in Wisconsin and used to assess the effectiveness of the Baljet assay for use in screening at a larger scale. Additionally, Oka498065

(PI 498065) (Bamberg et al. 2016) (48 hills from individual seeds), Oka458367 (PI 458367) (Bamberg et al. 2016) (48 hills from individual seeds), and the adapted Phureja (43 hills from individual seeds) were planted under the same conditions. Leaf disks were collected from three upper leaves of each plant using a standard handheld paper hole punch. The three leaf disks from each plant were placed in corresponding wells on a 96 deep-well plate, with each plate replicated in duplicate. The leaves in the 96-well plates were then lyophilized (Labconco, MO) for transportation to AAFC. Upon arrival at AAFC, the lyophilized samples were stored at room temperature overnight for analysis on the Baljet assay. Detailed information on germplasm used in this study can be obtained from the United States Potato Genebank (USPG) using the USDA-ARS Germplasm Resources Information Network (GRIN) (https://npgsweb.ars-grin.gov/gringlobal/search.aspx) or by visiting www.ars.usda.gov/midwest-area/madison-wi/vegetable-crops-research/people/john-bamberg/genebank-holdings/.

Development of Baljet Assay for Potato

Leaves

A handheld paper hole punch was used to produce four leaf disks from fresh plant leaflets of *S. okadae* (OKA15) and *S. tuberosum* cv. Kennebec plants grown in the greenhouse. Each species was replicated in triplicate for a total of twelve leaf disks. Four leaf disks of a species were placed in a single well of a flat-bottom 96 micro-well plate. Three wells were left blank to use as a negative control. Freshly prepared sodium picrate (200 uL) solution (9.5 mL 1% w/v picric acid, 0.5 mL 50% w/v NaOH) (Fisher Scientific, Ottawa, ON, Canada) was added to each well, including the negative control. The plate was sealed using aluminum seal tape (Thermo Fisher Scientific, Rochester, NY, USA) and vortexed for approximately 15 s. After vortexing, the plate was left to sit for 5 min before vortexing for another 15 s. Once the final vortexing was complete, the aluminum seal was removed. Using a pair of forceps, the leaf disks were removed from the plate and disposed of. The forceps were cleaned with water and wiped dry between wells. Wells containing a shift in colour from yellow to orange were indicative of the presence of lactone-containing compounds, such as cardiac glycosides. The endpoint absorbance was read on a plate reader (BioTek Synergy HTX Multimode Reader, Agilent Technologies Canada, Inc., Mississauga, ON, Canada) at 490 nm. The average absorbance (490 nm) and standard deviation was recorded (Table 1). Absorbance values for "Kennebec" and "OKA15" were compared to the sodium picrate negative control ("Control") using a one-way ANOVA and a post-hoc Tukey test. Significant differences at $p \leq 0.01$ and 0.05 were noted.

Wild common milkweed plants grown naturally outdoors were harvested and frozen at -20 °C before the foliar tissue was lyophilized (Labconco, MO) for three days to use as a cardiac glycoside standard on the Baljet assay. Foliar tissue from *S. okadae* (OKA15) plants grown in the greenhouse were harvested and processed in an identical manner to assess the amount of lactone-containing compounds produced in its foliar tissue – relative to milkweed – and to compare its absorbance with fresh OKA15 foliar samples. Twelve leaf disks of each plant species were made using the lyophilized samples. The leaf disks of each species were separated into three wells on a 96 micro-well plate and ran on the Baljet assay as described. The average absorbance (490 nm) and standard deviation was recorded (Table 1). The absorbance of lyophilized OKA15 foliar tissue was compared to that of fresh OKA15 using a two-sample t-test (Welch). The null hypothesis stated there was no difference between the absorbance of lyophilized and fresh foliar samples.

Table 1 Average A_{490nm} for the Baljet assay

	Number of Genotypes	Average	SD
Sodium Picrate	*	0.20	0.03
Ouabain (1 mM)	*	0.6	0.1
Kennebec (fresh)	1	0.30	0.07
OKA15 (fresh)	1	0.6	0.1
OKA15 (lyophilized)	1	0.67	0.04
Milkweed (lyophilized)	1	0.9	0.3
Oka x Phureja F_2Population	128	0.3	0.2
Oka458367	48	0.3	0.2
Oka498065	48	0.3	0.1
Adapted Phureja	43	0.33	0.08
Kennebec Tuber	1	0.08	0.03
OKA15 Tuber	1	0.05	0.02

*Not applicable

Tubers

Lyophilized OKA15 and *S. tuberosum* cv. Kennebec tubers were evaluated on the Baljet assay. Tuber cores (6 mm) for both species were collected from the cold storage supply at the Fredericton Research and Development Centre, AAFC and lyophilized (Labconco, MO). The tuber cores were ground (Geno/Grinder, SPEX SamplePrep, NJ) to a powder at 1500 rpm for 6 min in 50 mL Falcon tubes containing two ceramic beads. Ground tissue powder (0.025 g) was added to three QIAshredder tubes (QIAGEN, Toronto, ON, Canada) for each respective species. Three additional tubes did not contain tuber powder and were considered a sodium picrate control. Sodium picrate solution (200 uL) was added to the ground tissue and control tubes. The nine tubes were vortexed for 15 s and left to react for 5 min. After

the 5 min, the tubes were vortexed for another 15 s and then centrifuged (Eppendorf, Mississauga, ON, Canada). The QIAshredder tubes were centrifuged at a maximum speed (20817 rcf) for 4 min at room temperature to filter out debris and reduce viscosity of the solution. The supernatant from the filtrate in each tube was then transferred to a respective well on a flat-bottom 96 micro-well plate. The plate was centrifuged (246 rcf) for one minute at room temperature to remove any bubbles in the wells. The endpoint absorbance was read at 490 nm. The average absorbance (490 nm) and standard deviation was recorded (Table 1). Absorbance values for "Kennebec" and "OKA15" were compared to the sodium picrate negative control ("Control") using a one-way ANOVA and a post-hoc Tukey test. Significant differences at $p \leq 0.01$ and 0.05 were noted.

Field Trial Samples

Freshly prepared sodium picrate (200 uL) solution (9.5 mL 1% w/v picric acid, 0.5 mL 50% w/v NaOH) (Fisher Scientific, Ottawa, ON, Canada) was added to each well on the 96 deep-well plates with lyophilized leaves from the field trial. The plates were sealed and processed as previously described. After the final vortex, a multichannel pipette was used to transfer the sodium picrate solutions to a corresponding flat-bottom 96 micro-well plate for endpoint analysis. Wells containing a shift in colour were noted and the endpoint absorbance was read on a plate reader at 490 nm. The average absorbance (490 nm) of the F_2 population and standard deviation was recorded (Table 1). The frequency of absorbance was depicted in a histogram to select for outliers.

Ouabain

A 1:2 serial dilution of ouabain (Sigma-Aldrich, Oakville, ON, Canada), a commercially available cardiac glycoside, was prepared using 1 mM ouabain and distilled water to produce the following concentrations: 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.1 mM, and 0.01 mM. The 0.1 mM and 0.01 mM concentrations were included for consistency with feeding experiments. Sodium picrate (100 uL) – prepared as previously described – was added to 21 wells on a 96-well plate. Distilled water (100 uL) was added to three of the wells containing sodium picrate to produce a negative control. The ouabain solutions (100 uL) were added in triplicate to the plate using a multichannel pipette. The wells were mixed by pipetting and left to sit for 5 min. The endpoint absorbance was read at 490 nm and a spectral scan of the 1 mM solution was taken. The average absorbance (490 nm) and standard deviation was calculated for the 1 mM solution. A linear regression was performed using the absorbance on the Baljet assay as the dependent variable and the concentration of ouabain as the independent variable.

CPB Choice/No-Choice Feeding Assays

CPB feeding deterrence of *S. okadae* (OKA15) was evaluated using a choice/no-choice feeding assay as previously described (McCoy et al. 2022). Freshly laid CPB egg masses were obtained from laboratory colonies at the Fredericton Research and Development Centre of AAFC. Laboratory colonies were reared on *S. tuberosum* cv. Kennebec. The egg masses were left to hatch under 10-hour light and remained under these conditions until they reached the second instar (L2). The L2 larval stage for CPB demonstrated feeding preference behaviour in previous studies (Pelletier et al. 2001).

The amount of CPB feeding on leaves from *S. okadae* (OKA15) was compared to *S. tuberosum* cv. Kennebec, a susceptible species, using a feeding choice/no-choice assay. The assay was conducted using quantification of CPB feeding on leaf disks 1.2 cm in diameter. The disks were made using a metal cork-borer. Leaflets from the middle section of the plant were used to produce the disks. Disks were arranged following the template in Fig. 3 and each leaf disk overlapped another. The leaf area for disks was recorded prior to CPB feeding. L2 larvae were placed in either a Petri dish containing only *S. tuberosum* cv. Kennebec, only *S. okadae* (OKA15), or a dish containing leaf disks of both species. A single L2 larva was left to feed in each dish for 24 h under constant light. After the 24 h feeding period, the area of leaf consumed was quantified using LeafByte 1.3.0 (https://zoegp.science/leafbyte) and the percent of the original pre-feeding area

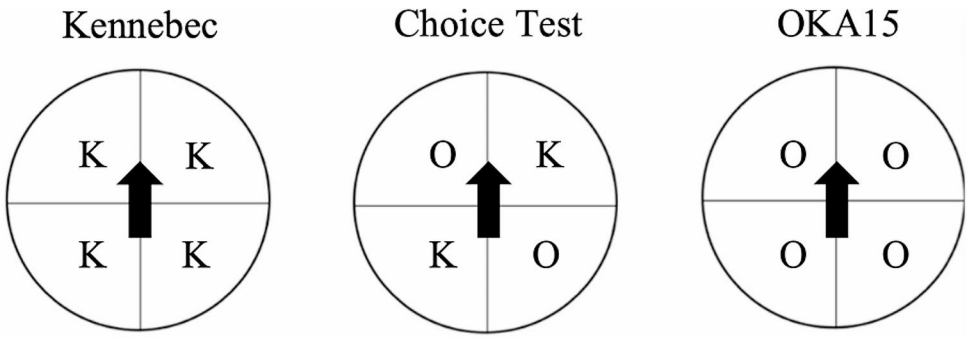

Fig. 3 Leaf disk placement of *S. tuberosum* cv. Kennebec ("K") and *S. okadae* (OKA15, "O") for the CPB choice/no-choice feeding assay. The arrow indicates the direction of the CPB larva head when placed in the Petri dish

for leaf disks were determined (Getman-Pickering et al. 2020; McCoy et al. 2022). There was a total of three replicate Petri dishes for each of the treatments ("Kennebec", "Choice Test", and "OKA15"). The percent leaf area consumed of leaf disk pairs was analyzed using a two-way ANOVA with species and treatment as factors, followed by a post-hoc Tukey test. Significant differences at $p \leq 0.01$ and 0.05 were noted.

A no-choice CPB feeding assay was also performed to investigate feeding deterrence from application of a commercially available cardiac glycoside (ouabain) and that of the neonicotinoid insecticide Titan® (active ingredient: clothianidin) currently used for crop management. The assay used 3 cm *S. tuberosum* cv. Kennebec leaf disks formed using a metal cork-borer. Large leaflets from the middle section of the plant were used to make the leaf disks. Using forceps, leaf disks were dipped in one of the following treatments: a distilled water control, a Titan® solution (0.62 ppm in distilled water, the reported LC_{90} for CPB) (Scott et al. 2023), or a 0.01 mM, 0.1 mM, or 1 mM solution of ouabain (Sigma-Aldrich, Oakville, ON, Canada) prepared with distilled water. Each treatment was replicated in triplicate. A single leaf disk was placed in fifteen 5 cm Petri dishes lined with moistened filter paper (Whatman 1, Millipore Sigma, Oakville, ON, Canada). Five L2 larvae were placed in each Petri dish and left to feed for 48 h under constant light. After the 48 h feeding period, the area of leaf consumed was quantified using LeafByte 1.3.0 (https://zoegp.science/leafbyte) and the percent of the original pre-feeding area for leaf disks were determined (Getman-Pickering et al. 2020). The percent area consumed for the different treatments were compared using a one-way ANOVA and a post-hoc Tukey test. Significant differences at $p \leq 0.01$ and 0.05 were noted.

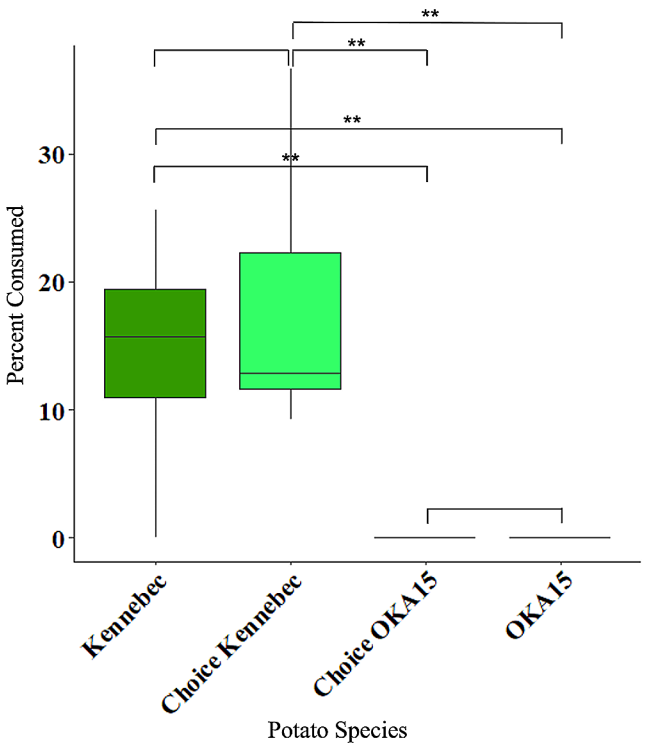

Fig. 4 Average percent area consumed of leaf disks in the no-choice ("Kennebec", "OKA15") and choice ("Choice Kennebec", "Choice OKA15") Petri dishes. The no-choice Petri dishes contained four leaf disks of either *S. tuberosum* cv. Kennebec or *S. okadae* (OKA15). The choice Petri dishes contained two leaf disks of each species. Significant differences between the percent area consumed of leaf disk pairs were identified using a two-way ANOVA and a post-hoc Tukey test and are indicated with ** ($p \leq 0.01$)

Results

S. okadae CPB Feeding Deterrence

L2 larvae in the choice Petri dishes – which contained leaf disks from both species – demonstrated a significant preference of feeding on *S. tuberosum* cv. Kennebec over *S. okadae* clone OKA15 (Fig. 4). When given the choice, the larvae consistently chose to avoid feeding on OKA15 and preferred Kennebec leaf disks. Comparisons between feeding on OKA15 and Kennebec leaf disks in the choice and no-choice treatments were all significantly different (Table S1, Table S2). There was no significant difference between the feeding on Kennebec in the choice dish ("Choice Kennebec") and the no-choice "Kennebec" dish ($p = 0.74$). There was also no significant difference between the feeding on the OKA15 in the choice dish ("Choice OKA15") and no-choice "OKA15" dish ($p = 1.0$). There was no feeding on OKA15 leaf disks in any of the Petri dishes. These results are consistent with previous studies comparing feeding on OKA15 with another cultivated variety, Shepody (McCoy et al. 2022).

CPB Ouabain Feeding Assay

The effectiveness of cardiac glycosides as a CPB feeding deterrent when applied externally to leaves was evaluated using a feeding assay. L2 larvae were provided *S. tuberosum* cv. Kennebec leaf disks dipped in either water, a CPB insecticide (Titan®), or a cardiac glycoside (ouabain) at 1 mM, 0.1 mM, or 0.01 mM for feeding. The larvae were left to feed for 48 h on the leaf disks to compare the percent area consumed of the treatments (Table S3, Table S4). After 48 h in the Petri dish, the larvae in the water controls had consumed the majority of the leaf disks (Fig. 5a). The larvae in the Titan® treatment had partially consumed the leaf disks (Fig. 5b). However, the leaf disks in the 1 mM ouabain treatment remained nearly untouched

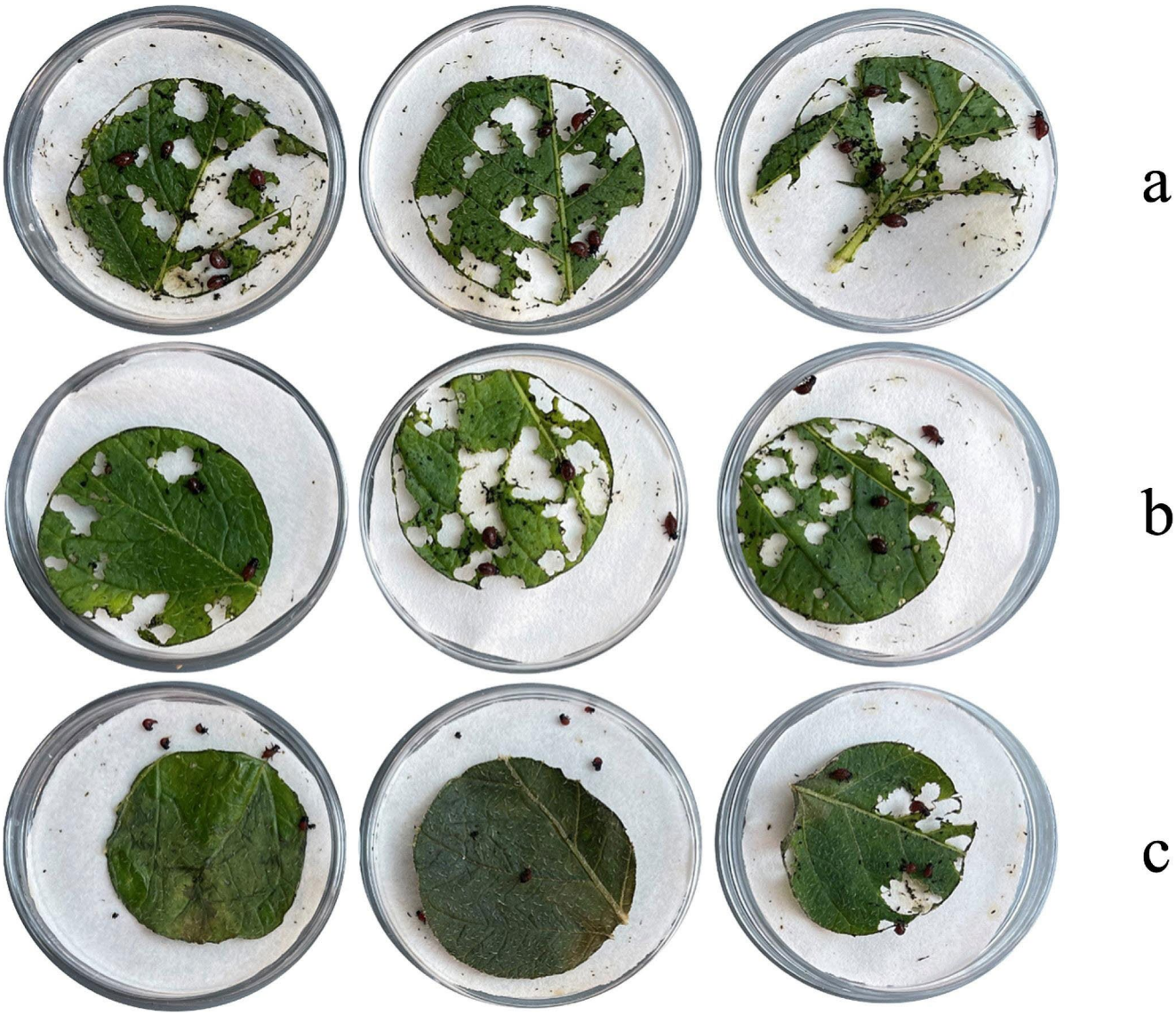

Fig. 5 *S. tuberosum* cv. Kennebec leaf disks dipped in a (**a**) water control, (**b**) neonicotinoid insecticide (Titan®, 0.62 ppm), or (**c**) cardiac glycoside (ouabain, 1 mM) after five L2 CPB larvae were left to feed in the Petri dishes for 48 h

and had less leaf area consumed than the water control. As shown in Figure S1, the 1 mM ouabain treatment had significant feeding deterrence when compared to the water control ($p = 0.018$), whereas the Titan® treatment did not ($p = 0.56$). The larvae in the water and Titan® Petri dishes appeared healthy as they were readily feeding, gaining mass, and producing ample frass. The larvae exposed to the cardiac glycoside, ouabain (1 mM), were not developing and had lack of movement and feeding. These signs of illness from exposure to 1 mM ouabain resulted in death for some larvae. Kennebec leaf disks treated with 0.01 mM and 0.1 mM of ouabain did not display significant feeding deterrence when compared to the water control ($p = 0.99$, $p = 0.37$) (Fig. S2).

Development of Baljet Assay for Potato

Leaves

Fresh *S. tuberosum* cv. Kennebec and *S. okadae* (OKA15) leaflets were tested using the Baljet assay (Fig. 6). The absorbance of the sodium picrate solutions treated with Kennebec and OKA15 leaf disks were compared to a sodium picrate control (Table S5, Table S6). The OKA15 leaf disks were found to produce a significantly higher absorbance than both the control ($p = 0.00029$) and Kennebec leaves ($p = 0.0014$), indicating the presence of lactone-containing compounds in its foliar tissue. There was no significant difference between the Kennebec and sodium picrate control ($p = 0.15$).

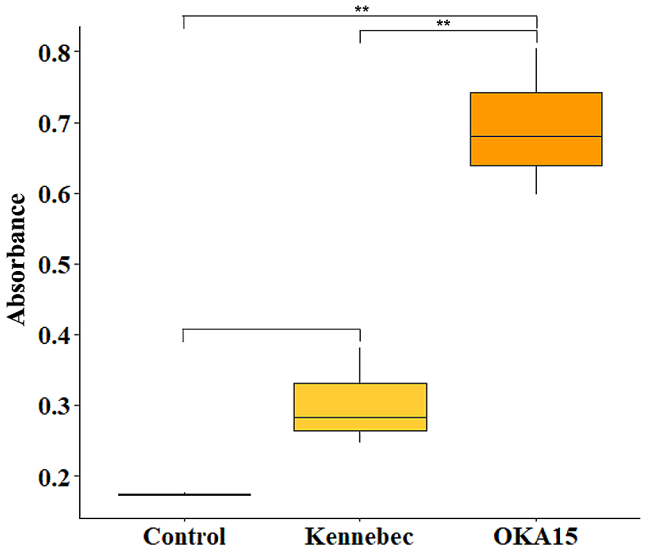

Fig. 6 Absorbance of Baljet reagent treated with *S. okadae* (OKA15) and *S. tuberosum* cv. Kennebec leaf disks at 490 nm in comparison to a sodium picrate control. Significant differences were identified using a one-way ANOVA and a post-hoc Tukey test and indicated with ** ($p \leq 0.01$)

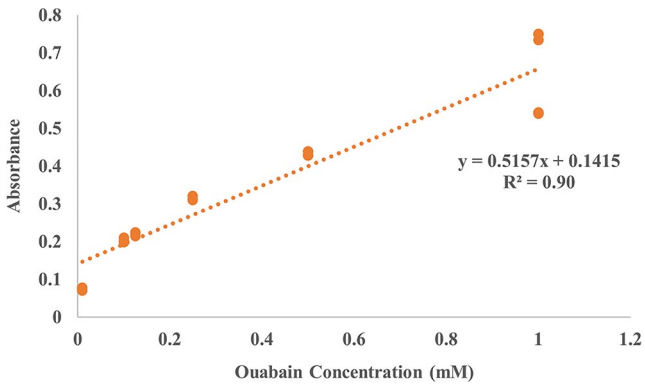

Fig. 7 Linear regression of the absorbance for varying ouabain concentrations on the Baljet assay at 490 nm

Lyophilized common milkweed was assessed on the described Baljet assay for use as a standard. Milkweed produced a high average absorbance of 0.9 ± 0.3 at 490 nm. Moreover, the ability of the Baljet assay to be used on fresh foliar samples was assessed by comparing lyophilized and fresh OKA15 foliar samples. The average absorbance of lyophilized OKA15 was found to be 0.67 ± 0.04, which was not significantly different than the fresh OKA15 foliar sample with an average absorbance of 0.6 ± 0.1 ($t_{(10)} = -0.94$, $p = 0.37$) (Table 1).

Tubers

Lyophilized *S. tuberosum* cv. Kennebec and *S. okadae* (OKA15) tuber powder was assessed for putative cardiac glycosides on the Baljet assay. The absorbance of the sodium picrate solutions treated with Kennebec and OKA15 tubers were compared to a sodium picrate control (Table S7, Table S8). As shown in Figure S3, there was no significant difference between OKA15 and the control ($p = 1.0$), or Kennebec and the control ($p = 0.26$). There was also no significant difference between the OKA15 and Kennebec ($p = 0.26$).

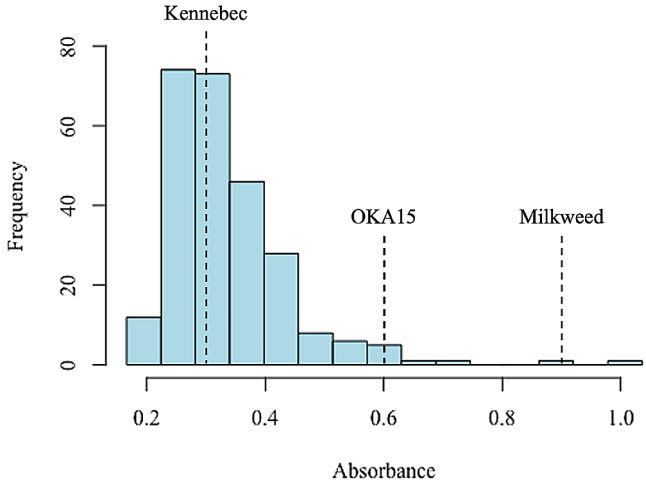

Fig. 8 Distribution of absorbance (490 nm) values from screening a field population of 128 individual *S. okadae* intermated F_2 progeny in duplicate for putative cardiac glycosides using the described Baljet assay. The average absorbance of fresh *S. tuberosum* cv. Kennebec (0.30), *S. okadae* (OKA15) (0.6), and lyophilized milkweed (0.9) foliar tissue are indicated for reference

Ouabain

A ouabain dilution series was used to determine an approximate threshold for the absorbance on the Baljet assay. The absorbance (A_{490nm}) of varying ouabain concentrations on the Baljet assay were fitted to a linear regression with an R^2 of 0.90 (Fig. 7). A significant colour change from yellow to orange was visible for the 1 mM ouabain solution that produced an average absorbance of 0.6 ± 0.1 at the desired wavelength.

Field Trial

A field trial containing intermated F_2 *S. okadae* × *S. tuberosum* Group Phureja (adapted Phureja) progeny was planted at the Hancock Agricultural Research Station (Hancock, Wisconsin). The leaves were used for quantification of putative cardiac glycosides using the Baljet assay to screen for potential CPB resistance on a larger scale. Samples were collected from the 128 plants in duplicate. The absorbance values for individuals in the population were plotted in a frequency distribution and assessed for outliers with high absorbance (Fig. 8). The screening results of intermated F_2 progeny from *S. okadae*× *S. tuberosum* Group Phureja

(adapted Phureja) demonstrated that there is a range of putative cardiac glycoside levels with an average absorbance of 0.3 and standard deviation of 0.2. The highest value was 1.028 and the lowest was 0.166. *S. tuberosum* Group Phureja (adapted Phureja) had an average absorbance of 0.33 with a standard deviation of 0.08. The result for *S. tuberosum* Group Phureja (adapted Phureja) was in the same range as the CPB susceptible *S. tuberosum* cv. Kennebec (with an average of 0.30 ± 0.07). The Baljet assay showed that OKA15 had a level of lactones that was higher than the average for *S. okadae*. There were other clones in the *S. okadae* accession that had higher A_{490nm} readings showing that levels of lactone-containing compounds are varying in populations of *S. okadae*.

Development of an Assay for Breeding

As indicated in Table 1, the leaflets used for the Baljet assay do not need to be freeze dried beforehand. The OKA15 fresh leaf disks had a comparable absorbance to that of the lyophilized OKA15 foliar tissue. This discovery makes it possible to rapidly screen potato plots in the field. The A_{490nm} for OKA15 foliage on the Baljet assay is lower than that of milkweed, a species known to produce multiple cardiac glycosides. Table 1 indicates an absorbance on the Baljet assay greater than 0.6 was found in OKA15 leaves capable of deterring CPB, whereas susceptible Kennebec leaves had a low absorbance of 0.30. In addition, 1 mM ouabain had an average absorbance of 0.6 on the Baljet assay and produced a similar feeding deterrence when applied to leaf disks. Analysis of leaf disks from an F_2 population of *S. okadae* plants indicated that an absorbance of 0.6 was higher than the average but was found in several individuals. Additional analysis on OKA15 tubers indicated no lactone-containing compounds were present in the material. These results indicate that selection of *S. okadae* clones for high levels of leaf-specific lactone-containing compounds for use as parents in breeding for CPB resistance is feasible. Not all accessions of *S. okadae* produce high levels of lactones and selection using the Baljet assay is recommended.

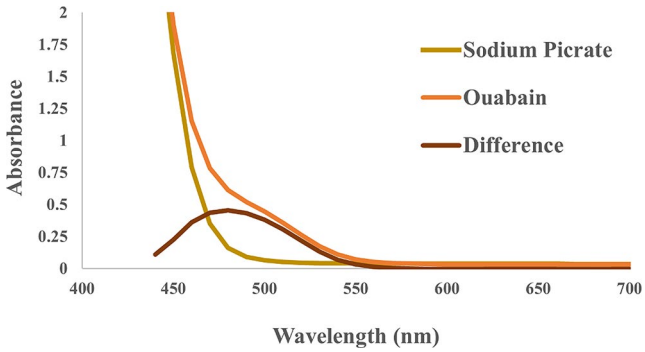

Fig. 9 Spectral scans of the average absorbance of sodium picrate and a commercially available cardiac glycoside, ouabain (1 mM), in sodium picrate. Sodium picrate has a maximum absorbance at approximately 430 nm. The difference of the two spectrums displays the characteristic presence of a peak around 490 nm indicating a Meisenheimer complex has formed from the reaction of sodium picrate with a cardiac glycoside

Discussion

The results show that the genotypes in the accession of *S. okadae* examined in this study have a range of distribution in the level of leaf-specific lactone-containing compounds, which includes possible cardiac glycosides, present in the foliage. The *S. okadae* clone, OKA15, was derived from this accession and it showed strong CPB deterrence and high foliar production of lactone-containing compounds. CPB deterrence was found for other clones in the accession in both the field and laboratory settings (Pelletier et al. 2001; McCoy et al. 2022). Other studies have reported that the *S. okadae* species in general had varying CPB resistance with some accessions showing high resistance (Flanders et al. 1992; Pelletier et al. 2001). In the current study, CPB demonstrated preferential feeding on the foliage of the susceptible cultivated potato variety, Kennebec, compared to OKA15. This is consistent with previous findings comparing feeding deterrence of OKA15 to the potato variety Shepody (McCoy et al. 2022). When not given the choice, CPB have consistently avoided feeding on OKA15 to the extreme of starvation (McCoy et al. 2022). Previous studies on the metabolites in OKA15 responsible for this CPB behaviour have identified putative cardiac glycosides in the foliage of OKA15 plants as a possible mechanism of resistance in potato (McCoy et al. 2022). The cardiac glycoside ouabain has been shown to inhibit the Na^+/K^+-ATPase of CPB (McCoy et al. 2022). In the current study we demonstrate that when applied externally to leaves, ouabain deterred CPB feeding in comparison to water and was more effective than the commercially available insecticide, Titan® (Fig. 5). The results showed that CPB consumption of foliar tissue was deterred in leaves containing putative cardiac glycosides produced naturally or treated externally with ouabain. These results provide further evidence for cardiac glycoside deterrence of CPB.

The Baljet assay developed to screen for lactone-containing compounds, such as cardiac glycosides, in potato relies on an indicative absorbance around 490 nm (Korchagina and Petrova 1979). The traditional Baljet assay has been used on both *Digitalis* leaves and medicinal extracts containing cardiac glycosides (Neuwald 1950). A spectral scan (Fig. 9) of the Baljet assay solution containing the cardiac glycoside ouabain demonstrates the characteristic absorbance corresponding to the Meisenheimer complex produced during

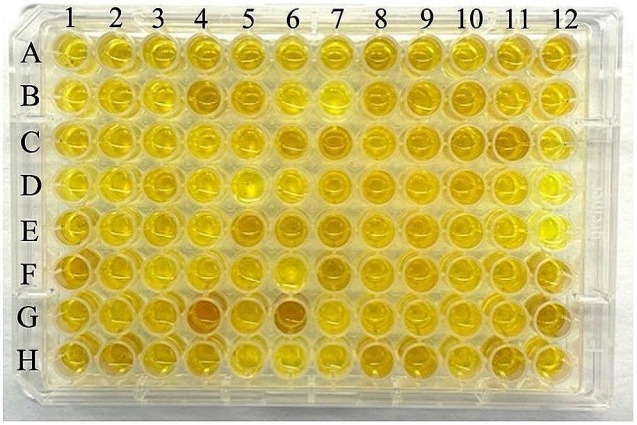

Fig. 10 Example of visually screening the Baljet assay results from the *S. okadae* field trial. Each well contained a sodium picrate solution treated with leaf disks from a single progeny. Leaf disks were removed from the plate before analysis on a plate reader. Dark orange wells indicate the presence of lactone-containing compounds, like cardiac glycosides. Wells D12 and E12 were a negative sodium picrate control

the assay (Fig. 2) (Korchagina and Petrova 1979). Sodium picrate in an alkaline medium has a maximum absorbance at approximately 430 nm (Fig. 9). Once a cardiac glycoside is introduced to a solution of sodium picrate, an additional absorbance emerges at approximately 490 nm. The appearance of a peak at 490 nm is responsible for the change in colour from yellow to orange. The change in absorbance at 490 nm can be analysed using a plate reader for statistical purposes (Figs. 6 and 8) or inspected visually for rapid screening (Fig. 10).

Production of putative cardiac glycosides in potato foliage was a novel finding and presented a new biological target to combat CPB (McCoy et al. 2022). Cardiac glycosides are not commonly found in the *Solanum* genus. Some members of the family of Solanaceae plants, which *Solanum* is a part of, have been noted to produce cardiac glycosides (Pomilio et al. 2008). In addition, Solanaceae plants were reported to produce withanolides – a class of compounds containing a steroidal lactone similar to those found in cardiac glycosides (Evans et al. 2009). The lactone ring of withanolides would also be detected on the Baljet assay. However, there was no detection of withanolides in previous LC-MS analyses of *Solanum* species (McCoy et al. 2022), indicating the variation observed in this study is most likely due to differing levels of cardiac glycoside biosynthesis. Studies on the biosynthesis of glycoalkaloids in *Solanum* suggest that divergence in biosynthetic pathways utilizing sterols can allow for the production of cardiac glycosides in addition to glycoalkaloids – which are the most abundant foliar secondary metabolite in *Solanum* species – as a defence mechanism to prevent herbivory (Bennett and Wallsgrove 1994; Sonawane et al. 2018; Erb and Kliebenstein 2020).

The production of leaf-specific cardiac glycosides in potato will allow breeders to develop CPB resistant varieties from germplasm derived from *S. okadae*. The Baljet assay reported herein is not only a very rapid and cost-effective assay, but it employs widely available chemicals and can be performed in the field. Utilization of breeding germplasm with *S. okadae* in their pedigree, along with screening with the Baljet assay, provides an efficient way to select for CPB resistance earlier in breeding programs. Future work will also include using the Baljet assay to screen genetic mapping populations to find genes associated with lactone production in potato foliage. Purification and structural determination of the lactone-containing bioactive compound(s) from *S. okadae* foliar tissue is underway.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12230-024-09963-5.

Acknowledgements The authors thank Isabelle Gemmill and Ghaith Zamzam for technical support. The experiments comply with the current laws of the country in which they were performed.

Author Contributions The authors confirm contribution to the paper as follows: study conception and design: McCoy HJ, Tai HH, MacKinley P, Vickruck J; creation of F_2 field population: Bamberg JB; data collection: McCoy HJ, Fenstemaker S; analysis and interpretation of results: McCoy HJ, Tai HH, Fenstemaker S, Bamberg JB, MacKinley P, Vickruck J, Calhoun LA; draft manuscript preparation: McCoy HJ, Tai HH; obtained funding for the project: Tai HH. All authors reviewed the results and approved the final version of the manuscript.

Funding Project funding was from AAFC A-base funds. HJM was supported by the David M. Armstrong Doctoral Fellowship and New Brunswick Innovation Foundation (NBIF) PhD recruitment award awarded through the University of New Brunswick.
Open access funding provided by Agriculture & Agri-Food Canada library.

Data Availability The data underlying this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing financial interest.

References

Ainembabazi, D., X. Geng, N. S. Gavande, J. J. Turchi, and Y. Zhang. 2022. Synthesis and biological evaluation of Cardiac glycosides for Cancer Therapy by targeting the DNA damage response. *Chemmedchem* 17: e202200415. https://doi.org/10.1002/cmdc.202200415

Akinmoladun, A. C., M. T. Olaleye, and E. O. Farombi. 2014. Cardiotoxicity and Cardioprotective Effects of African Medicinal Plants. In *Toxicological Survey of African Medicinal Plants*, 395–421. Elsevier. https://doi.org/10.1016/B978-0-12-800018-2.00013-3

Alyokhin, A., G. Benkovskaya, and M. Udalov. 2022. Chapter 4 - Colorado potato beetle. In *Insect Pests of Potato*, Second Edition, 29–43. Academic Press. https://doi.org/10.1016/B978-0-12-821237-0.00027-5

Bamberg, J., A. Del Rio, and D. A. Navarre. 2016. Intuitive visual impressions (cogs) for identifying clusters of diversity within Potato species. *American Journal of Potato Research* 93: 350–359. https://doi.org/10.1007/s12230-016-9508-6

Bejček, J., M. Jurášek, V. Spiwok, and S. Rimpelová. 2021. *Quo Vadis Cardiac Glycoside Research? Toxins* 13: 344. https://doi.org/10.3390/toxins13050344

Bennett, R. N., and R. M. Wallsgrove. 1994. Secondary metabolites in plant defence mechanisms. *New Phytologist* 127: 617–633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x

Cingel, A., J. Savić, J. Lazarević, T. Ćosić, M. Raspor, A. Smigocki, and S. Ninković. 2016. Extraordinary adaptive plasticity of Colorado Potato Beetle: Ten-Striped Spearman in the era of Biotechnological Warfare. *International Journal of Molecular Sciences* 17: 1538. https://doi.org/10.3390/ijms17091538

Erb, M., and D. J. Kliebenstein. 2020. Plant secondary metabolites as defenses, regulators, and primary metabolites: The blurred functional trichotomy. *Plant Physiology* 184: 39–52. https://doi.org/10.1104/pp.20.00433

Evans, W. C., D. Evans, and G. E. Trease. 2009. *Trease and Evans pharmacognosy*. 16th ed. Edinburgh; New York: Saunders/Elsevier.

Flanders, K. L., J. G. Hawkes, E. B. Radcliffe, and F. I. Lauer. 1992. Insect resistance in potatoes: Sources, evolutionary relationships, morphological and chemical defenses, and ecogeographical associations. *Euphytica* 61: 83–111. https://doi.org/10.1007/BF00026800

Fox, J., and S. Weisberg. 2019. *An R companion to Applied Regression (version third edition)*. Thousand Oaks CA: Sage.

Friedman, M. 2006. Potato glycoalkaloids and metabolites: Roles in the plant and in the Diet. *Journal of Agricultural and Food Chemistry* 54: 8655–8681. https://doi.org/10.1021/jf061471t

Getman-Pickering, Z. L., A. Campbell, N. Aflitto, A. Grele, J. K. Davis, and T. A. Ugine. 2020. LeafByte: A mobile application that measures leaf area and herbivory quickly and accurately. Edited by Satu Ramula. *Methods in Ecology and Evolution* 11: 215–221. https://doi.org/10.1111/2041-210X.13340

Haviv, H., and S. J. D. Karlish. 2013. P-Type Pumps: Na+,K+-ATPase. In *Encyclopedia of Biological Chemistry*, 681–687. Elsevier. https://doi.org/10.1016/B978-0-12-378630-2.00198-5

Haynes, K. G., L. Zotarelli, C. T. Christensen, and S. Walker. 2019. Early generation selection within a diploid hybrid *Solanum tuberosum* groups Phureja and Stenotomum population for the intense yellow-fresh creamer potato market. *Hortscience* 54(12): 2118–2124. https://doi.org/10.21273/HORTSCI13576-18

Kaiser, N., N. C. Manrique-Carpintero, C. DiFonzo, J. Coombs, and D. Douches. 2020. Mapping *Solanum chacoense* mediated Colorado potato beetle (Leptinotarsa decemlineata) resistance in a self-compatible F2 diploid population. *Theoretical and Applied Genetics* 133(9): 2583–2603. https://doi.org/10.1007/s00122-020-03619-8

Kassambara, A. 2023. ggpubr: ggplot2 Based Publication Ready Plots. R package (version 0.6.0).

Korchagina, G. T., and E. A. Petrova. 1979. Physicochemical methods of determining cardiac glycosides of the strophanthidin series (review). *Pharmaceutical Chemistry Journal* 13: 970–977. https://doi.org/10.1007/BF00777672

McCoy, H. J., C. Zeng, E. McCoy, P. MacKinley, J. Vickruck, L. A. Calhoun, and H. H. Tai. 2022. Evidence for Cardiac glycosides in Foliage of Colorado Potato Beetle-Resistant *Solanum okadae*. *Journal of Agricultural and Food Chemistry* 70: 14613–14621. https://doi.org/10.1021/acs.jafc.2c04311

Morsy, N. 2017. Cardiac Glycosides in Medicinal Plants. In *Aromatic and Medicinal Plants - Back to Nature*, ed. Hany A. El-Shemy. InTech. https://doi.org/10.5772/65963

Mukherjee, P. K. 2019. Bioactive Phytocomponents and Their Analysis. In *Quality Control and Evaluation of Herbal Drugs*, 237–328. Elsevier. https://doi.org/10.1016/B978-0-12-813374-3.00007-7

Neuwald, F. 1950. The Photometric assay of Digitoxin by the Baljet reaction. *Journal of the American Pharmaceutical Association (Scientific ed)* 39: 172–174. https://doi.org/10.1002/jps.3030390314

Neuwirth, E. 2022. RColorBrewer: ColorBrewer Palettes. R package (version 1.1-3).

Norn, S., and P. R. Kruse. 2004. [Cardiac glycosides: From ancient history through Withering's foxglove to endogeneous cardiac glycosides]. *Dansk Medicinhistorisk Arbog*: 119–132.

Paudel, J. R., K. M. Gardner, B. Bizimungu, D. De Koeyer, J. Song, and H. H. Tai. 2019. Genetic mapping of Steroidal glycoalkaloids using selective genotyping in Potato. *American Journal of Potato Research* 96: 505–516. https://doi.org/10.1007/s12230-019-09734-7

Pelletier, Y., C. Clark, and G. C. Tai. 2001. Resistance of three wild tuber-bearing potatoes to the Colorado potato beetle. *Entomologia Experimentalis et Applicata* 100: 31–41. https://doi.org/10.1046/j.1570-7458.2001.00845.x

Pomilio, A. B., E. M. Falzoni, and A. A. Vitale. 2008. Toxic Chemical compounds of the Solanaceae. *Natural Product Communications* 3: 1934578X0800300. https://doi.org/10.1177/1934578X0800300420

R Core Team. 2023. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Scott, I. M., J. Vickruck, S. Hann, S. Krolikowski, P. MacKinley, J. Stokes-Rees, G. Hatten, and C. Moffat. 2023. Regional differences in susceptibility to spinosyn insecticides registered for Colorado potato beetle management in Canada. *Pesticide Biochemistry and Physiology* 193: 105459. https://doi.org/10.1016/j.pestbp.2023.105459

Sinden, S. L., L. L. Sanford, W. W. Cantelo, and K. L. Deahl. 1986. Leptine glycoalkaloids and Resistance to the Colorado Potato Beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense*. *Environmental Entomology* 15: 1057–1062. https://doi.org/10.1093/ee/15.5.1057

Sonawane, P. D., U. Heinig, S. Panda, N. S. Gilboa, M. Yona, S. Pradeep Kumar, N. Alkan, T. Unger, S. Bocobza, M. Pliner, S. Malitsky, M. Tkachev, S. Meir, I. Rogachev, and A. Aharoni. 2018. Short-chain dehydrogenase/reductase governs steroidal specialized metabolites structural diversity and toxicity in the genus *Solanum*. *Proceedings of the National Academy of Sciences* 115. https://doi.org/10.1073/pnas.1804835115

Souza, E., K. F. C. Souza, B. P. Tavares Moraes, I. C. Nunes De Palmer, P. Paixão, A. Burth, Ribeiro Silva, and C. F. Gonçalves-de-Albuquerque. 2021. Na+/K+-ATPase as a target of Cardiac glycosides for the treatment of SARS-CoV-2 infection. *Frontiers in Pharmacology* 12: 624704. https://doi.org/10.3389/fphar.2021.624704

Tai, H. H., and J. Vickruck. 2022. Potato resistance against insect herbivores. In *Insect Pests of Potato*, 277–296. Elsevier. https://doi.org/10.1016/B978-0-12-821237-0.00014-7

Tai, H. H., K. Worrall, Y. Pelletier, D. De Koeyer, and L. A. Calhoun. 2014. Comparative metabolite profiling of *Solanum tuberosum* against Six Wild *Solanum* Species with Colorado Potato Beetle Resistance. *Journal of Agricultural and Food Chemistry* 62: 9043–9055. https://doi.org/10.1021/jf502508y

Tai, H. H., K. Worrall, D. De Koeyer, Y. Pelletier, G. C. Tai, and L. A. Calhoun. 2015. Colorado Potato Beetle Resistance in *Solanum oplocense* X *Solanum Tuberosum* Intercross hybrids and Metabolite markers for selection. *American Journal of Potato Research* 92: 684–696. https://doi.org/10.1007/s12230-015-9484-2

Wickham, H. 2016. *ggplot2: Elegant graphics for data analysis*. New York: Springer-.

Wickham, H., and J. Bryan. 2023. readxl: Read Excel Files. R package (version 1.4.2).

Wickham, H., R. François, L. Henry, K. Müller, and D. Vaughan. 2023. dplyr: A Grammar of Data Manipulation. R package (version 1.1.2).

Wierenga, J. M., and R. M. Hollingworth. 1992. Inhibition of insect acetylcholinesterase by the potato glycoalkaloid α-chaconine. *Natural Toxins* 1: 96–99. https://doi.org/10.1002/nt.2620010207

Wolters, P. J., D. Wouters, Y. M. Tikunov, S. Ayilalath, L. P. Kodde, M. F. Strijker, L. Caarls, R. Gf, Visser, and V. Gaa. 2023. Vleeshouwers. Tetraose steroidal glycoalkaloids from potato provide resistance against Alternaria solani and Colorado potato beetle. *eLife* 12: RP87135. https://doi.org/10.7554/eLife.87135.3

Yamane, H., K. Konno, M. Sabelis, J. Takabayashi, T. Sassa, and H. Oikawa. 2010. Chemical Defence and Toxins of Plants. In *Comprehensive Natural Products II*, 339–385. Elsevier. https://doi.org/10.1016/B978-008045382-8.00099-X

Yencho, G. C., S. P. Kowalski, G. G. Kennedy, and L. L. Sanford. 2000. Segregation of leptine glycoalkaloids and resistance to Colorado potato beetle (Leptinotarsa decemlineata (say)) in F2*Solanum tuberosum* (4x) ×*S. chacoense* (4x) potato progenies. *American Journal of Potato Research* 77: 167–178. https://doi.org/10.1007/BF02853941