Project report for SSCR-Potatoes

***Project title*:**

Control of cyst nematodes by characterisation and manipulation of the biosynthesis of the egg hatching compounds

Applicant(s):

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Background to the project

Plant parasitic nematodes are estimated to diminish global agricultural output by approximately 10%, which has been conservatively estimated to be valued at >$125 billion (Chitwood, 2003). These pests are difficult to detect in soil and their identification is difficult due to their conservative morphology. Their control is likely to become more problematic in the future due to increases in soil temperatures associated with climate change, loss in nematicides due to concerns about their environmental toxicity (EU regulation EC 1107/2009), further spread due to increasing world trade in agricultural commodities and pressures on agricultural land to increase food production for an increase global population. Novel management methods are needed to minimise damage from these pests and to control their spread. In this project, genotypic variation in potato for production of nematode hatching factor will be investigated in order to determine whether manipulation of hatching factor synthesis can provide "non-hosts" or alternatively hatching factor factories for use in "suicide hatching". The development of a microfluidic platform for improving throughput in nematode hatching assays will also be a focus of this project.

Aims and objectives

1) Develop novel microfluidic methods to increase throughput of nematode hatching bioassays.
2) Optimize potato root leachate capture and fractionation methods and hatching factor compound analysis.
3) Identify, propagate and cross potato genotypes with a wide range of concentrations of hatching factor compounds.
4) Undertake comparative transcript profiling of potato genotypes identified as containing high and low levels of hatching factor compounds.

Research results

1. We have optimised an egg-hatching protocol for high throughput robust comparison of the biological efficacy of root exudates from different cultivars: Our initial experiments which utilized hatching from *Globodera rostochiensis* cysts in response to both tomato and potato (*Solanaum tuberosum* 'Desirée') root exudates using established methods, showed wide batch variation (Figure 1). This was circumvented by utilizing isolated eggs and optimized for high throughput analysis. The in-house protocol provides for egg isolation and fabrication of micro devices as depicted in Figure 2. These devices enable an *in vitro* mimic of population-based hatching from cysts. The data from these assays cautioned against the utility of a microfluidic device designed around single egg capture due the egg-to-egg variability in intrinsic hatching efficacy (Figure 3) and therefore the mesh-based device was adopted as standard. An important progression facilitated by these devices was the ability to screen for chemical induction of hatching with a view to identifying a standard benchmark against which to compare the hatching efficacy of root exudates. This highlighted the ATP mimic sodium metavanadate which outperforms all natural exudates and is now routinely used as a highly reproducible positive control for egg hatching (Figure 4). The biological basis for the action of metavanadate is unknown: understanding this has potential to provide fundamental insights into the molecular mechanisms underpinning hatching and is an interesting side-project emanating from the research. Data are reported in 3 meeting abstracts. Completion of this objective enables routine and robust screens for hatching efficacy of root exudates versus the metavanadate benchmark.

2. We have standardized protocols to obtain root exudates with defined hatching efficacy. This has enabled biochemical characterisation of exudates with high (H) and low (L) hatching efficacy and resolved chemical components consistently present in high efficacy exudate. Root exudate is prepared in the pipeline depicted Figure 5. This protocol was initially applied to 8 distinct cultivars grown and extracted in Dundee. The results (Figure 6) led to the observation that hatching efficiency from 8 genetically distinct cultivars showed clear variation that was initially hypothesized may reflect the distinct chemical composition of the different cultivar exudates. The recent focus switched to comparing hatching from a restricted set of cultivars including *Solanaum tuberosum* 'Desirée' (Tetraploid), *S. tuberosum* 'Atlantic' (Tetraploid) and S. *violaceimarmoratum* (Diploid). Interestingly even when analysing consecutively produced batches of the same exudates we obtained extremes of hatching efficacy between 'high hatching' (Batch 12) and 'low hatching' (Batch 13) compared to the metavanadate benchmark. We systematically characterised lyophilisation as a robust route for concentrating large volume exudates that retain bioactivity. This allows for the concentration of chemicals and their detection by (HPLC) and mass spectrometry (Figure 7). Analysis of the 'Desirée' exudate identified 'α Chaconine' (Figure 8 A). Importantly, sequentially produced root exudates from Desirée, Atlantic and Vio showed different hatching efficacies such that all cultivars from batch 12 were HIGH (H) and from 13 LOW (L): This provides an excellent opportunity for comparative chemical analysis to resolve constituents selectively present in H exudate. We made the striking observation that major peaks common to each H exudate were missing from the L exudates (Figure 9). Our first pass analysis utilized predicative chemical composition based on mass charge of the peaks resolved by HPLC. In the case of the major peak found in the H exudates this indicates a list of 2 major components of defined by parent and fragmented ions (Figure 8 A and B). These candidates, α chaconine and α solanine, are known significant constituents of root exudates. The minor peaks are currently under investigation. In parallel, commercially available pure compounds will be tested for hatching efficacy to identify bona fide hatching factors.

3. Our observations and further analysis of other components associated with exudates with high hatching activity will provide a panel of root exudate components that can be screened for composition to inform on the strategies discussed in aim 3.

4. The approaches described in 1 and 2 identify that even between distinct batches from the same cultivar that there are exudates from similarly grown plants that give H and L hatching efficacy. As this difference, when measured with reliable hatching assays highlights differing bioactivity it indicates that the mRNA from these plants may reveal gene expression difference that drive these differences. Plant material from batches 12 (H) and 13 (L) have been collected and frozen to allow this comparison. We are holding on this until we have a better understanding of the batch differences and deciding the requisite expertise that has been lost from the supervisory team can be found elsewhere.

Outcomes

1) Develop novel microfluidic prototypes for improved efficiency in counting nematodes for use in hatching factor assays.
2) Optimize potato root leachate isolation methods and characterization of potato genotypes with a wide variation in hatching factor compounds.
3) Identify candidate genes regulating biosynthesis of hatching factor compounds.
4) Develop genotypes producing high level of hatching factor for use in commercial hatch factor extraction.
5) Identify / develop high hatching factor resistant varieties for planting to help clear fields of PCN by promoting hatching in the absence of an available food source.

At this point in the project it has delivered an optimised protocol for high throughput hatching assays that has been adopted by our broader research group and is impacting on mode of action studies for crop protection agents. This has been presented at national meetings and there has been interest in its application. It forms the basis of two manuscripts in preparation.

The comparative analysis of root exudates with high and low hatching efficacy has provided a biological and biochemical characterisation of plant tissue samples. These samples have been stored and will serve as a starting point to investigate the genetic basis of hatching factor biosynthesis.

Next steps

As outlined above, the focus of the project will be resolving chemical components of biologically characterised root exudates from a selected range of cultivars as the next step towards understanding the biosynthesis of hatching factors.

Appendix

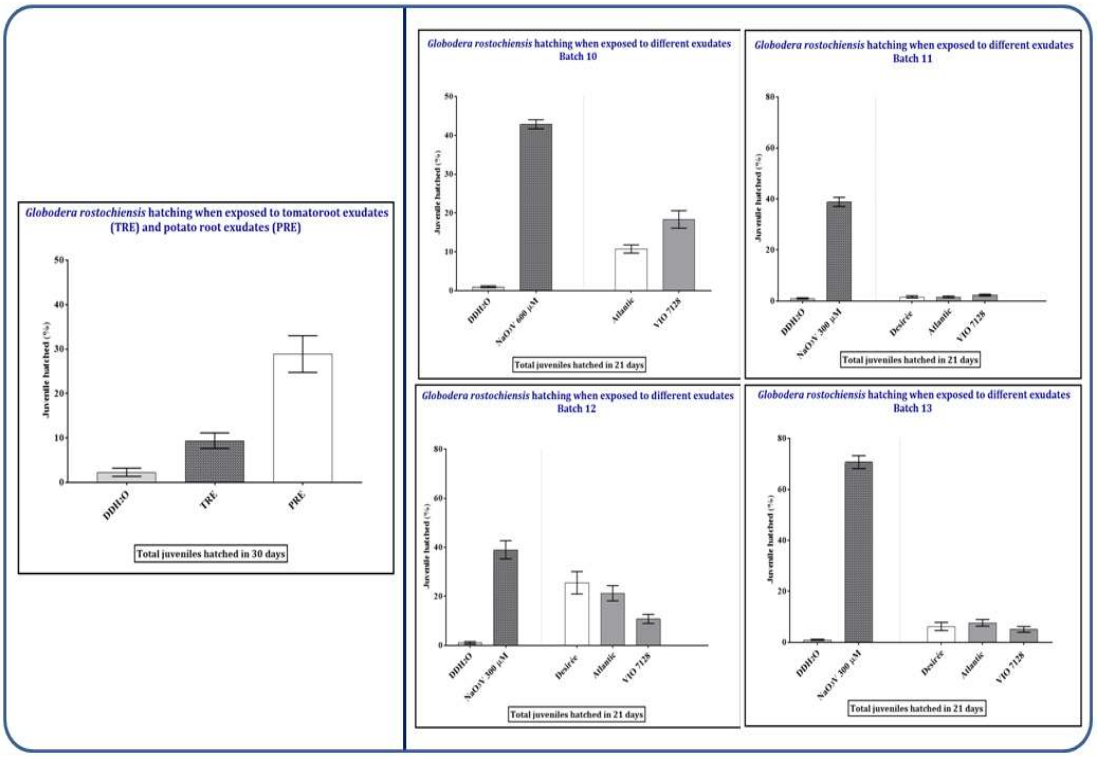


Figure 1: Variability in *G. rostochiensis* hatching from cysts when exposed to different batches of root exudates. Each hatching assay had three replicates, incubated at 20^{o}C for three weeks. The assay plates were sealed with parafilm and placed inside a plastic container lined with moist tissue paper which was placed inside the incubator. The tissue paper inside the container was moistened every day. The test exudates or chemicals were changed once a week on the 7th, 14th and 21st day. At the end of the experiment, the juvenile hatch was expressed as % of total hatched juveniles/ 500 (approx.) isolated eggs x 100.

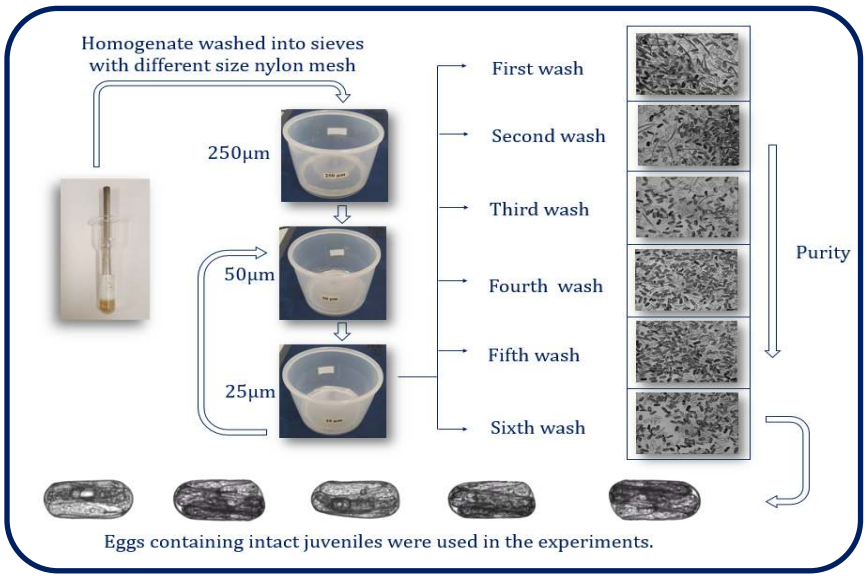


Figure 2: Preparation of isolated purified egg inoculum. Cyst were crushed in a glass homogenizer and washed with ultrapure water in sieves with different size mesh. 250µm sieve separated the cyst fragments from the mixture of juveniles and eggs whereas most of the juveniles entangled in the middle 50µm sieve were separated from mixture of eggs after washing. Six repetitive washes (left) between 50 and 25µm mesh separated most of the juveniles from the mixture of eggs. At the end, egg inoculum with mostly encapsulated potentially viable eggs and minimal empty, disintegrated eggs and juveniles were collected into a beaker from the bottom sieve.

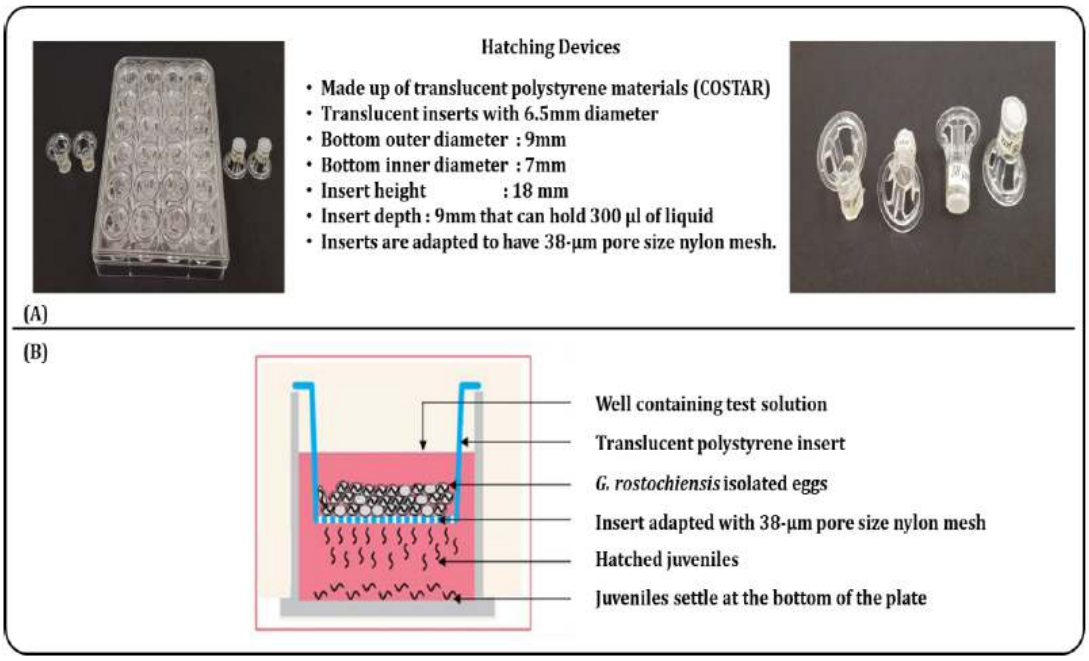


Figure 3 **: Hatching devices.** (A) Hatching devices (inserts) were made from translucent polystyrene materials with 7 mm bottom diameter, 18mm height and 9 mm deep that can hold 300 µl of suspension. The inserts were adapted to have 38µm pore size nylon mesh. (B) Diagrammatic representation of functioning of hatching device. Insert holding isolated eggs were lodged in each well of culture plates containing indicated amount of root exudates. Upon stimulation by the exudates once the juvenile hatched, they passed through the mesh and settled at the bottom of the plate.

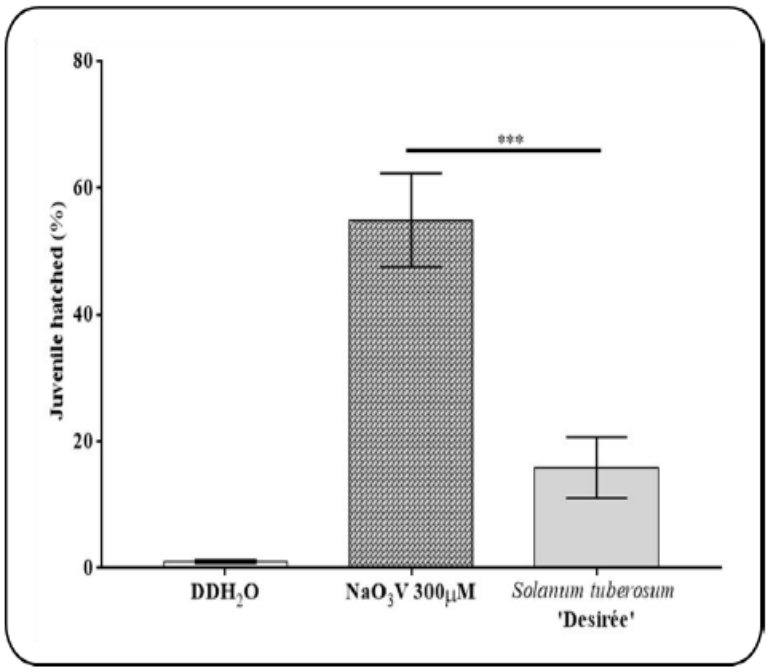


Figure 4: *Globodera rostochiensis* hatching when exposed to potato root exudates (Desirée) and sodium metavanadate (NaO_3V). Egg based hatching assay with NaO_3V as a benchmark was conducted to match it against established root exudates prepared from *Solanum tuberosum* (Desirée). Juvenile hatch was expressed as % of total hatched juveniles/ 500 isolated eggs x 100.

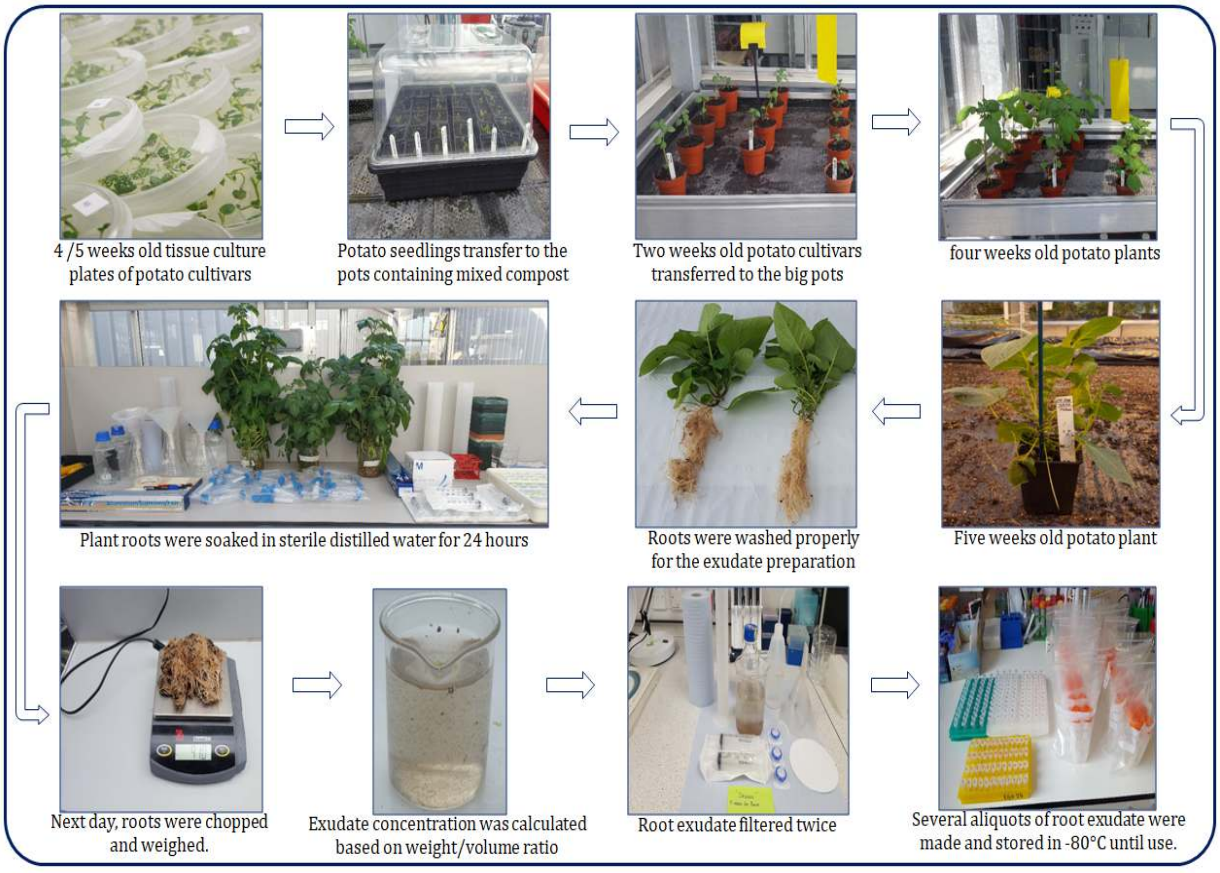


Figure 5: Systematic representation of root exudate preparation. Four to five weeks old plant cuttings of different cultivars in tissue culture plates were used as the plant source for growing potatoes. Cultured cuttings were transferred in plastic pots containing compost and grown for five

to seven weeks. For exudate extraction, roots of individual plants from each variety were washed gently. Plants from each variety were pooled and the roots from the intact plants were submerged in a beaker containing DDH_2O. Exudates were collected from the root system of each cultivar for 24 hours. After exudate extraction, the entire volume of water the roots were soaked in was measured and roots were cut at the root- shoot junction, patted dry and weighed. The final concentration was calculated according to weight per volume ratio. Exudates were filtered twice to remove soil debris and microorganisms. Root exudates from different cultivars were normalized by diluting to fixed weight per volume ratio and were routinely at 20 g wet weight root per litre of DDH_2O. Root exudates were stored as aliquots at -80^oC until use.

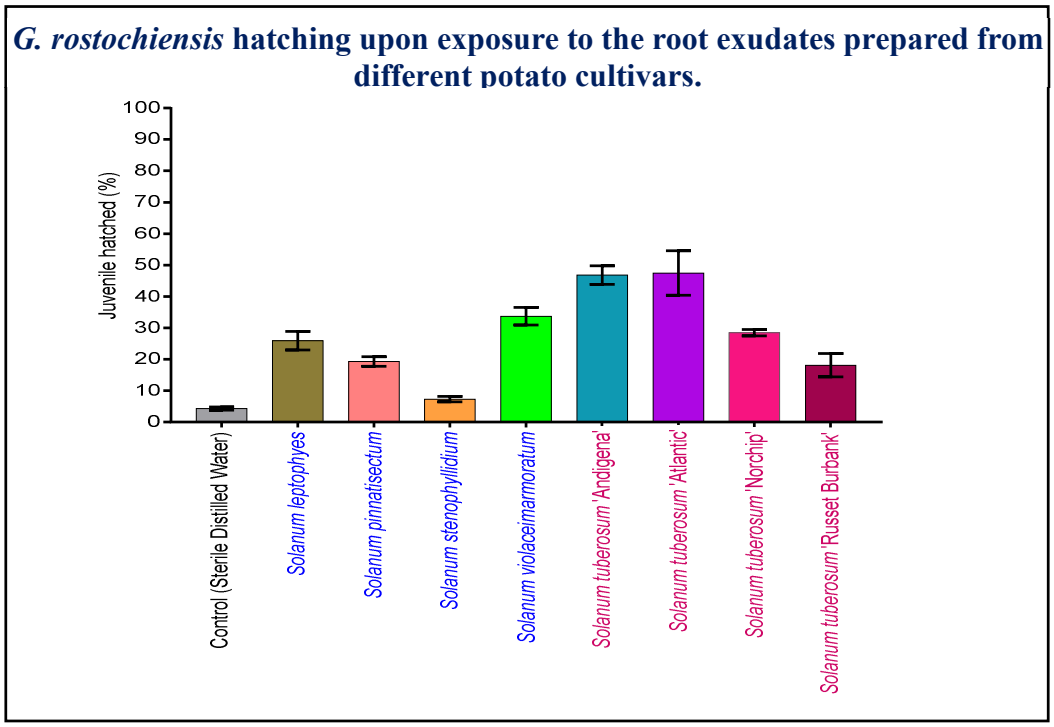
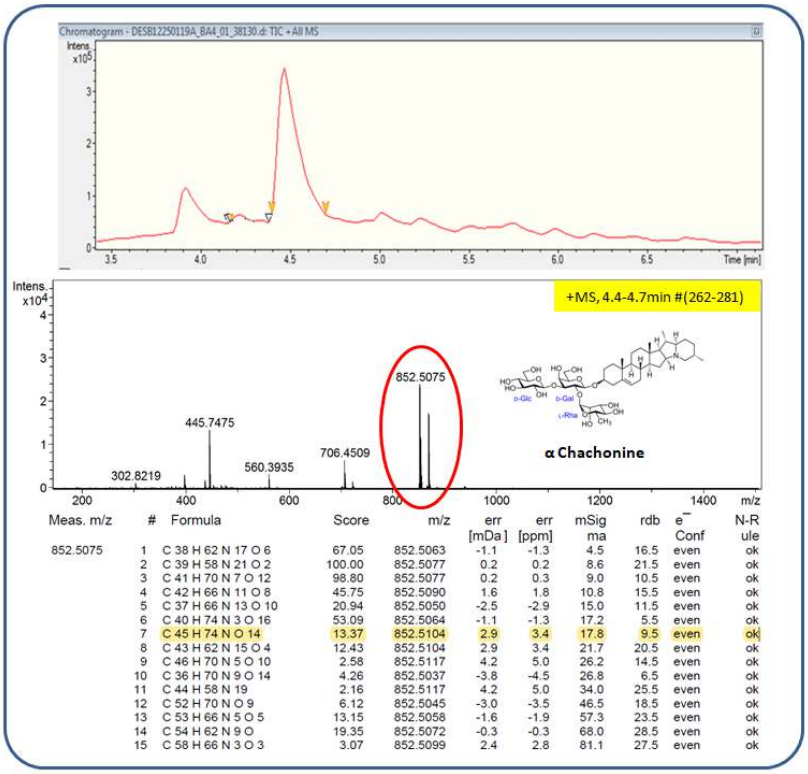


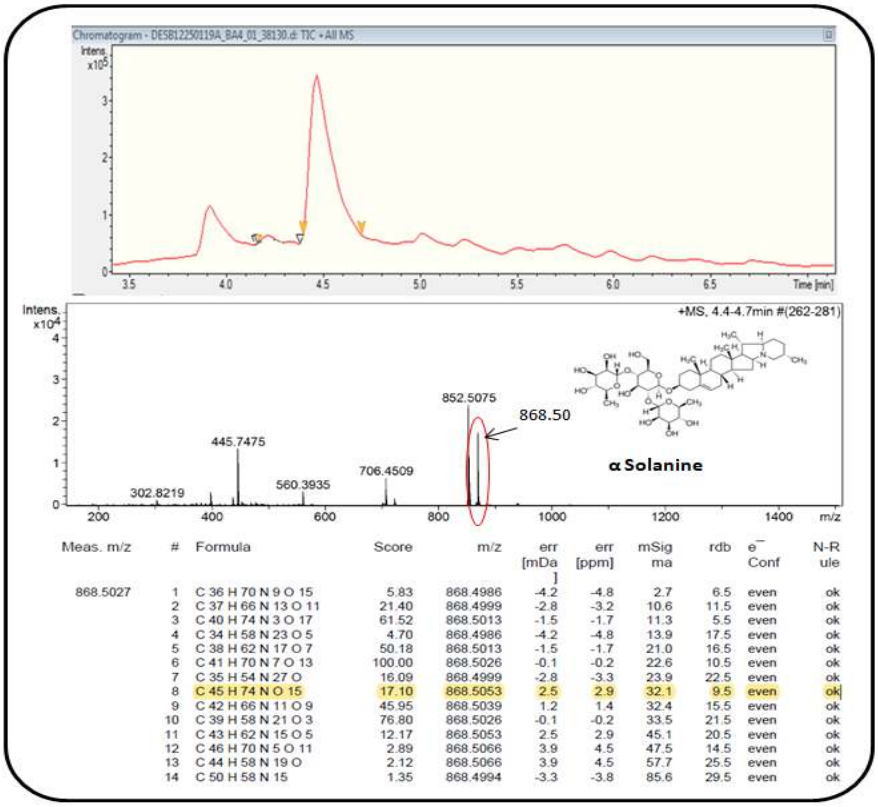
Figure 6:* G. rostochiensis *hatching upon exposure to the root exudates prepared from different potato cultivars. Hatching assay had three replicates, incubated at 20^oC for three weeks. The assay plates were sealed with parafilm and placed inside a plastic container lined with moist tissue paper which was placed inside the incubator. The tissue paper inside the container was moistened every day. The test exudates were changed once a week on the 7th, 14th and 21st day. At the end of the experiment, the juvenile hatch was expressed as % of total hatched juveniles/ 500 (approx.) isolated eggs x 100.

Figure 7: Mass spectrometry. Samples were analysed using a MaXis (Bruker Daltonics, Bremen, Germany) time of flight (TOF) mass spectrometer. Samples were introduced to the mass spectrometer via a Dionex Ultimate 3000 autosampler and uHPLC pump and variable wavelength detector. Ultrahigh performance liquid chromatography was performed using a Waters UPLC BEH C18 (50 mm x 2.1 mm 1.7µm) column with 4 UV wavelengths selected. Gradient elution from 5% acetonitrile (0.2% formic acid) to 100% acetonitrile (0.2% formic acid) was performed in ten minutes at a flow rate of 0.6 ml/min. High resolution positive ion electrospray ionisation mass spectra were recorded



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSig ma	rdb	e⁻ Conf	N-R ule
852.5075	1	C 38 H 62 N 17 O 6	67.05	852.5063	-1.1	-1.3	4.5	16.5	even	ok
	2	C 39 H 58 N 21 O 2	100.00	852.5077	0.2	0.2	8.6	21.5	even	ok
	3	C 41 H 70 N 7 O 12	98.80	852.5077	0.2	0.3	9.0	10.5	even	ok
	4	C 42 H 66 N 11 O 8	45.75	852.5090	1.6	1.8	10.8	15.5	even	ok
	5	C 37 H 66 N 13 O 10	20.94	852.5050	-2.5	-2.9	15.0	11.5	even	ok
	6	C 40 H 74 N 3 O 16	53.09	852.5064	-1.1	-1.3	17.2	5.5	even	ok
	7	C 45 H 74 N O 14	13.37	852.5104	2.9	3.4	17.8	9.5	even	ok
	8	C 43 H 62 N 15 O 4	12.43	852.5104	2.9	3.4	21.7	20.5	even	ok
	9	C 46 H 70 N 5 O 10	2.58	852.5117	4.2	5.0	26.2	14.5	even	ok
	10	C 36 H 70 N 9 O 14	4.26	852.5037	-3.8	-4.5	26.8	6.5	even	ok
	11	C 44 H 58 N 19	2.16	852.5117	4.2	5.0	34.0	25.5	even	ok
	12	C 52 H 70 N O 9	6.12	852.5045	-3.0	-3.5	46.5	18.5	even	ok
	13	C 53 H 66 N 5 O 5	13.15	852.5058	-1.6	-1.9	57.3	23.5	even	ok
	14	C 54 H 62 N 9 O	19.35	852.5072	-0.3	-0.3	68.0	28.5	even	ok
	15	C 58 H 66 N 3 O 3	3.07	852.5099	2.4	2.8	81.1	27.5	even	ok

Figure 8(A): **Mass spectrometry of Desirée;** note the detection of α-chachocine which has previously been reported to have hatching efficacy and serves as proof of concept for the experimental approach



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
868.5027	1	C 36 H 70 N 9 O 15	5.83	868.4986	-4.2	-4.8	2.7	6.5	even	ok
	2	C 37 H 66 N 13 O 11	21.40	868.4999	-2.8	-3.2	10.6	11.5	even	ok
	3	C 40 H 74 N 3 O 17	61.52	868.5013	-1.5	-1.7	11.3	5.5	even	ok
	4	C 34 H 58 N 23 O 5	4.70	868.4986	-4.2	-4.8	13.9	17.5	even	ok
	5	C 38 H 62 N 17 O 7	50.18	868.5013	-1.5	-1.7	21.0	16.5	even	ok
	6	C 41 H 70 N 7 O 13	100.00	868.5026	-0.1	-0.2	22.6	10.5	even	ok
	7	C 35 H 54 N 27 O	16.09	868.4999	-2.8	-3.3	23.9	22.5	even	ok
	8	C 45 H 74 N O 15	17.10	868.5053	2.5	2.9	32.1	9.5	even	ok
	9	C 42 H 66 N 11 O 9	45.95	868.5039	1.2	1.4	32.4	15.5	even	ok
	10	C 39 H 58 N 21 O 3	76.80	868.5026	-0.1	-0.2	33.5	21.5	even	ok
	11	C 43 H 62 N 15 O 5	12.17	868.5053	2.5	2.9	45.1	20.5	even	ok
	12	C 46 H 70 N 5 O 11	2.89	868.5066	3.9	4.5	47.5	14.5	even	ok
	13	C 44 H 58 N 19 O	2.12	868.5066	3.9	4.5	57.7	25.5	even	ok
	14	C 50 H 58 N 15	1.35	868.4994	-3.3	-3.8	85.6	29.5	even	ok

Figure 8(B): Mass spectrometry of Desirée: note the detection of α-solanine which has also previously been reported to have hatching efficacy and further validates the experimental approach.

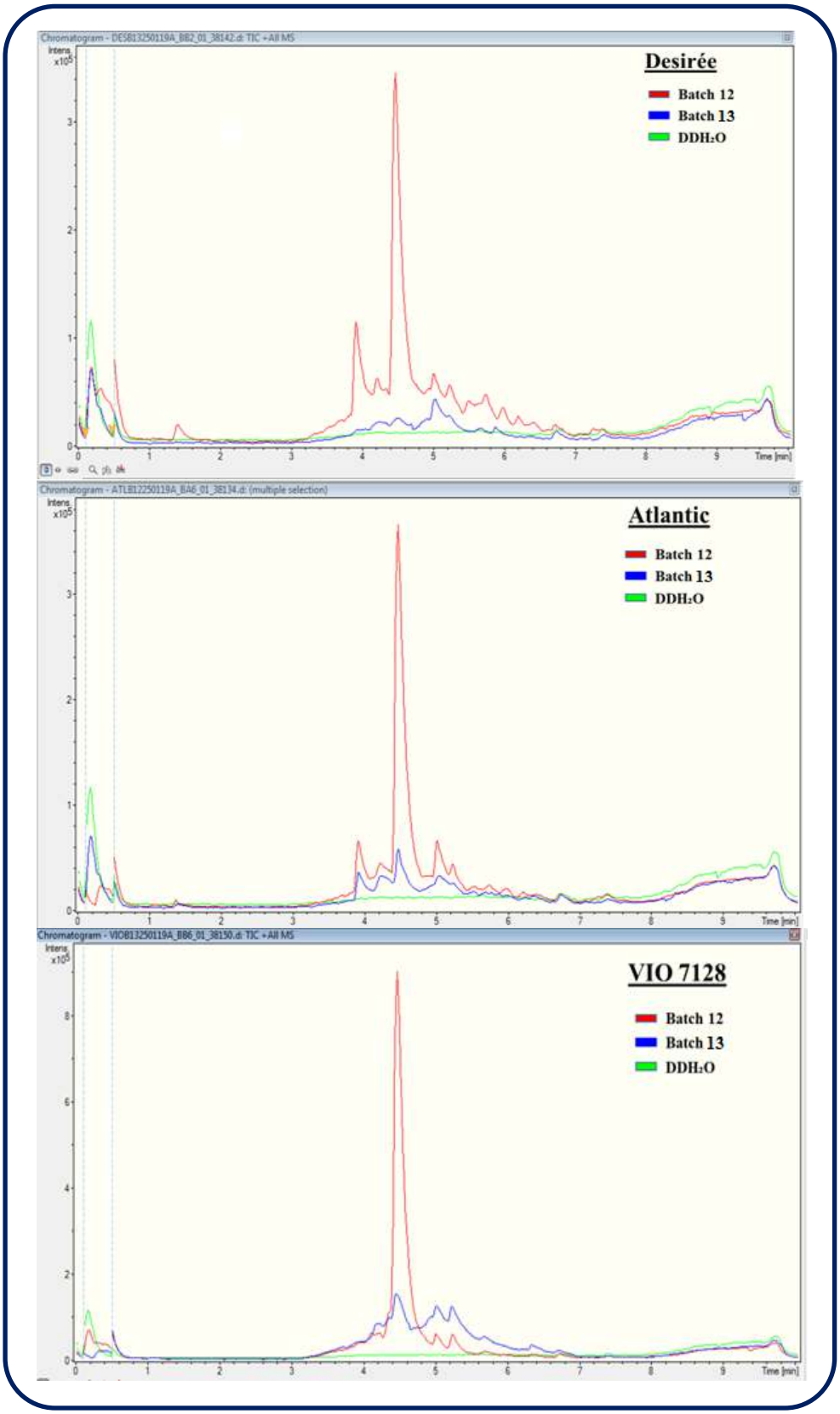


Figure 9: Mass spectrometry of different root exudates with differential hatching efficacies from distinct batches with High and Low hatching Differential profiles for batch 12, high hatching, versus batch 13, low hatching. Note the consistent profile between all the cultivars. Several differential peaks are resolved which will be subject to further characterisation.