Solanoeclepin A

Characterization of a rhizosphere communication molecule in tomato and potato

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Chapter 3

Comprehensive analysis of the *Solanum* section *Petota* reveals widespread solanoecllepibin A production and novel insight into potato cyst nematode hatching

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Abstract

*Globodera pallida* and *Globodera rostochiensis* are known as potato cyst nematodes (PCNs), obligate endoparasites that infect *Solanaceous* plants. PCN eggs need to perceive hatching factors (HFs) from the host plant to start their life cycle. Solanoeclepin A (SolA) is so far identified as the most active HF for PCNs. In this work we analysed the presence of SolA in the root exudate (RE) of a collection of *Solanum* species belonging to the section *Petota*. We reveal large inter- and intra-species variation in the amount of SolA produced. Surprisingly, there is no linear relationship between SolA level and the hatching rate of PCN, possibly as a result of hatching inhibitors. However, hatching assays with RE from non-SolA producing accessions also demonstrates the presence of other hatching factors which induce hatching of *G. pallida* but not of *G. rostochiensis*. Our results demonstrate that hatching is a complex process determined by the genetics of both the host and the PCN species. Our study paves the way to the discovery of new HFs in potato.

Keywords

Hatching assay; Potato; Potato Cyst Nematode; root exudate; solanoeclepin A
Introduction

Cyst nematodes (CNs) are obligate plant-parasitic nematodes with negative influences on crop yield and agriculture. The diversity of CNs is reflected in their taxonomy, consisting of seven genera and 110 species with varying degrees of host specificity. This specificity is thought to be defined by the co-evolution of CN species and their hosts, some of which are for example apple, bamboo, cabbage, chickpea, maize, rice, soybean, eggplant, tomato and potato (Subbotin et al. 2010). The prolonged survival of cysts in agricultural soils and overcoming of the resistance genes in current cultivars are two of the major issues in the fight against these devastating pests. In addition, most of the nematicides, such as soil fumigants, are now banned (Oka 2020) and current management practices therefore necessarily include long periods of crop rotation or fallow. Together this results in CNs being a large problem for farmers.

Potato is one of the crops suffering the most from the CN problem (Turner and Subbotin 2013), with potato cyst nematodes (PCNs), *Globodera pallida* and *Globodera rostochiensis*, being responsible for 9% of all potato yield losses in Europe alone. Both species are a serious threat to solanaceous species (International Potato Center 2020). Both the Solanaceae and PCNs originated from the Andes in South America, one of the most biodiverse regions of the world where potato and tomato speciation also occurred (Jenkins 1948; International Potato Center 2020). Due to their worldwide distribution, PCNs are a quarantine pest in more than 100 countries around the world (EPPO A2 List 2021).

Despite their diversity in host range, CNs share a similar life cycle that begins with the hatching of the egg, followed by migration of the hatched larvae towards a suitable host, penetration of the host root, formation of a feeding site and three cycles of moulting (from J2 to J3, J4 and finally adult life stages). Adult males regain motility, exit the root and find the females for fertilization of the eggs. Impregnated females swell and die, which results in the formation of a resistant quiescent structure called cyst (Subbotin et al. 2010; Perry et al. 2018). The cyst thus is the hardened body of the dead female containing hundreds of eggs. Cysts remain in the soil when the plants are harvested but may also be dispersed through contaminated agricultural products, for example seed tubers, across large distances. Cysts protect the embryonated eggs against biotic and abiotic stresses for up to 20 years (Perry et al. 2018).

When a suitable host is nearby, a hatching factor (HF) produced by the host roots is perceived by the quiescent eggs within the cyst and hatching takes place. In a population of eggs, some J2 (usually <10% of all eggs) can spontaneously hatch without the perception of HFs. Another proportion of the eggs in a population may not hatch immediately upon the perception of the HF but may require two or multiple exposures. This “bet hedging” behavior may be an important adaptation to unpredictable environments and has likely been positively selected for through evolution as was suggested for other, animal, parasitic nematodes such as *Nematodirus battus* (van Dijk and Morgan 2010). The perception of HFs from an appropriate host triggers stylet and
muscle movement in the J2s which finally results in rupturing of the eggshell and release of the pre-parasitic J2 (Mkandawire et al. 2022). Hatched J2s move towards the host guided by the perception of multiple host signals (Ochola et al. 2020). One of the strategies pursued to eradicate CNs is the employment of compounds that stimulate hatching in the absence of the host, a strategy called ‘suicide hatching’ (Ngala et al. 2021).

Despite all the research conducted on CN hatching, particularly in PCNs, it is noteworthy that only a few natural HFs have been isolated and characterized in host plant RE. The first HF, for soybean cyst nematode *Heterodera glycines*, was identified by Masamune et al. in 1982 in kidney bean root and was coined glycinoeclepin A, a triterpenoid acid. In 1985, Fukuzawa et al. isolated another two HFs from the same species with a similar chemical structure to glycinoeclepin A, which were thus called glycinoeclepin B and C. Another hatching factor reported in the literature is solanoeclepin A (SolA) discovered in 1966 by Mulder et al. in potato RE and later also in tomato, which acts as the principal HF of PCNs (Schenk et al. 1999; Guerrieri et al. 2021). There are other hatching stimulating compounds present in the RE of potato and tomato; Shimizu et al. (2020) showed that steroidal glycoalkaloids (SGAs) also induce hatching of *G. rostochiensis* cysts. The most active SGA produced by potato is solasonine inducing 80% hatching at a concentration of 5 µM. However, SolA is much more active, inducing 90% hatching at a concentration of about 2 pM (Shimizu et al. 2020a; Guerrieri et al. 2021).

SolA has also been detected in the RE of Solanaceae other than potato and tomato such as *Solanum sisymbriifolium*, *Solanum pimpinellifolium* and *Solanum habrochaites* (Guerrieri et al. 2021). However, up to now very little information is known about the presence of SolA let alone its variation in wild relatives of potato. According to the International Potato Center (CIP) there are more than 4000 varieties of native potatoes grown in the Andean highlands of Peru, Bolivia and Ecuador (International Potato Center 2020). The large diversity of wild potato relatives belonging to the genus *Solanum* in the section *Petota*, is mainly due to the easy hybridization of many species, which can be a problem for taxonomists, but a helpful resource for breeders and botanists (Jacobs et al. 2011). Indeed, these wild relatives of potato are a precious resource for valuable traits to study and improve the resistance of cultivated potato to PCNs (Jacobs et al. 2008). So far, understanding the resistance mechanisms to PCNs has focused on the parasitic stage and hardly any attention has been paid to the pre-parasitic stage, the hatching process. Exploring the natural variation in HF production and understanding how HFs contribute to the hatching process using these wild relatives of potato will potentially offer novel solutions for PCN resistance.

To explore the potential of this approach, we analysed SolA production in the RE of more than 300 accessions from the Petota section and found considerable variation in the SolA level, including genotypes that did not produce any detectable SolA. Subsequently we assessed the hatching activity on *G. pallida* and *G. rostochiensis* in a selection of low and high SolA producers. Interestingly, REs from several of the null SolA accessions
significantly stimulated hatching of *G. pallida*, but not of *G. rostochiensis*. This suggests the presence of putative novel HFs with different specificity for *G. pallida* and *G. rostochiensis*. Finally, based on the findings presented here, we reflect on the evolution of SolA and related HFs in the Solanaceae and on the HF specificity in PCN in light of their adaptation and co-evolution with compatible host species.

**Materials and methods**

**Chemicals and materials**

An authentic standard of solanoeclepin A was kindly provided by prof. Keiji Tanino (Hokkaido University, Japan). The methanol, acetonitrile, deionized water and formic acid for UPLC-MS/MS and LC-ESI-QTOF-MS analysis were all hyper grade for LC-MS, purchased from Biosolve BV, The Netherlands. Deionized (Milli-Q) water was prepared using a water purification system Milli-Q® (Merck Millipore, Burlington, MA, USA).

**Plant materials and growing conditions**

From the in vitro collection of Wageningen UR Plant Breeding (Vleeshouwers et al. 2011) a subset of 340 genotypes (Suppl. Table S1) was used for the experiment. The set contained accessions of 128 tuber-bearing Solanum species. Three weeks after the in vitro plantlets were put on a rooting medium, they were transferred to 17 cm pots with potting compost and grown in a greenhouse of Unifarm (Wageningen), with a relative humidity (RH) between 50 and 90% (not controlled), light minimum of 16h, and a set minimum temperature of 18/20°C. (N/D). Plants were watered daily with 0.5 x Hoagland for six weeks before collection of samples. In the experiments where plants were regrown, five cuttings were put per pot and five replicates (pots) were grown.

**Sample collection, extraction and purification**

RE collection was performed on six weeks old plants: distilled water was poured onto the soil and the flow through from the pot was collected in a beaker until 500 ml was reached. For sample processing, the RE was filtered over filter paper to remove soil particles. 14 ml of root exudate was applied to a Discovery® DSC-18 SPE Tube (500 mg/6ml, Supelco, Bellefonte, PA, USA) column purchased from Sigma-Aldrich Co. LLC (Germany), pre-conditioned with one column volume (6 ml) of 100% methanol and activated with one column volume of deionized (Milli-Q) water. The retained sample was washed with one column volume of deionized (Milli-Q) water to remove salts, SolA elution from the cartridge was achieved with 3 ml of 100% methanol which was collected in a 4 ml glass vial. Samples were evaporated until dryness in vacuo and reconstituted with 150 µL of 20% methanol in water (v/v) for UHPLC-MS/MS analysis.

The extraction of SolA from the regrown plants was performed according to Guerrieri et al. 2021 using an SPE Oasis® MAX (3 cc/60mg, Waters, Milford, MA, USA) column.
**UHPLC-MS/MS analysis**
The analysis of SolA was performed as described by Guerrieri et al. (2021), using a Waters Acquity ultra-high pressure liquid chromatography (UHPLC)TM I-Class System (Waters) equipped with a binary solvent manager and sample manager, coupled to a Xevo® TQ-XS tandem quadrupole mass spectrometer (MS/MS, Waters MS Technologies, Manchester, UK) with electrospray (ESI) ionization interface. The instrument control, MS data acquisition, and processing were carried out by the MassLynxTM software, version 4.2 (Waters).

**Fractionation of RE**
Fractionation of RE was performed as described by Guerrieri et al. (2021). 1 ml of root exudate was freeze dried, salts were precipitated by dissolving the dried exudate in 1 ml of methanol. The sample was centrifuged at 4000 rpm for 3 min, and the supernatant was transferred to a clean vial prior to drying by vacuum evaporation. The sample was then re-dissolved in 150 µl 25% ACN and injected on UHPLC. Separation of the samples was achieved using the same gradient and same column as used for the analysis. The eluent was fractionated using a Waters fraction manager with each fraction spanning 30 s of the chromatogram.

**Nematode culturing and populations**
Two batches of the Dutch population D383 of *G. pallida* were used (Rouppe Van Der Voort et al. 1997). Batch 1-2A has been in use for more than 5 years while batch 1-3C is in use since the second half of 2018. For *G. rostochiensis*, cysts of the Dutch population Mierenbos were employed (Bakker and Bouwman-Smits 1988). Nematode culturing (mass propagation) was performed at Wageningen University and Research facility using an internal protocol. After acetonation, dried cysts can be stored at -80°C for prolonged periods. Batches of cysts are taken from the -80°C and placed at 4°C for at least 2 months before use to ensure breakage from diapause and only then are used for hatching assays.

**Hatching assays**
All cysts were hydrated using tap-water on a 100 µm metal sieve in a glass Petri dish for 7 days in tap water prior to exposure to REs. For hatching assays, the sieve containing the hydrated cysts was moved to a new glass Petri dish and placed under a stereomicroscope for dissection of the cysts using metal needles. Intact eggs were rinsed from the cysts through the sieve and kept in suspension by pipetting. From this egg suspension aliquots of 100 µl were taken and dispensed in wells of flat-bottomed 96 well Nunc™ MicroWell™ plates (ThermoScientific). The egg suspension was diluted or concentrated accordingly to obtain on average 40-60 eggs per well. Aliquots from each of the REs of the randomly selected accessions were diluted to 40% of their original concentration and 100 µl were inoculated on the wells containing PCN eggs. The REs were diluted five times to reach the final concentration. Five replicates
(wells) in a single plate were used for each root exudate tested. Given the number of samples to be tested, the mass screening of hatching of non-producer accessions was divided over 15 batches (plates) with 9 different REs and 3 controls per plate. The controls were always the same in every plate and consisted of tap water (negative control) and the REs of the two accessions with the (qualitatively) highest amounts of SolA, MF-II and KTZ 276-3, respectively. After a plate was completely set up, pictures of the entire wells were taken using a Leitz DM IRB inverted microscope (Leica) with a 2.5x objective and a mobile phone coupled to the ocular piece. These were named 0h pictures and were used for calculating the numbers of eggs and J2 per well at the start of the experiment. The hatching was assessed at 10 days after the beginning of the experiment. The hatching percentage was calculated according to the formula:

\[
\text{Hatching percentage} = \frac{\text{Number of J2s at 10d} - \text{Number of J2s at 0h}}{\text{Total number of eggs per well}} \times 100
\]

Data analysis

Hatched eggs were counted using photos of each well using the multipoint selection tool of ImageJ (Schneider et al. 2012). Raw values were uploaded to Excel and processed for statistical analysis using either R or GraphPad Prism. For the non-parametric analysis (PERMANOVA) of hatching the adonis function of the Vegan package (Oksanen et al. 2020) was used with the standard settings, except for the number of permutations for which a setting of 10000 was used. Observations with a value equal to 0% were manually changed to 0.1% due to the clustering model used.

We used the model

\[
P_{\text{hatch}} = A_i + B_j + O + N_{\text{eggs}} + e
\]

where A was the potato accession/treatment i tested (i was one of 131 potato cultivars, tap water, KTZ 276-3 or MF-II), B was batch j (1 – 15), O was the date of observation (10 days, except for batch 9, which was scored on day 11), N_{\text{eggs}} the number of eggs (95% interval: 24 – 148) and the residual variance e. As we found that batch (R2 = 0.00038; p = 0.49), date of observation (R2 = 0.00081; p = 0.18), and the number of eggs (R2 = 0.00140; p = 0.048) only captured a small amount of variance, no batch correction was conducted neither was the data filtered to remove outlying observations based on the number of eggs. Therefore, our model could be simplified to

\[
P_{\text{hatch}} = A_i + e
\]
To determine whether there were significant differences between accessions/treatments, a Kruskal-Wallis test was performed. Side-by-side (pairwise) differences were calculated using a Wilcoxon rank sum test. In this way 17,956 tests were performed. The obtained p-values were corrected for multiple testing by using the \textit{p.adjust} function with the false discovery rate (FDR) method (Benjamini and Hochberg 1995).

For the remaining hatching data analyses a two-way ANOVA (confirmation of top hatching non-producer accessions found in the mass screening of hatching) or a one-way, non-parametric ANOVA (hatching of \textit{G. rostochiensis} Mierenbos by selected non-producer \textit{Petota} accessions) were performed using the recommended post-hoc tests suggested by GraphPad Prism.

\textbf{Phylogenetic tree}

A NJ phylogenetic tree for the genotypes used in this study was constructed based on the AFLP data of (Jacobs et al. 2008) using the Manhattan similarity index implemented in PAST (Hammer et al. 2001).

\textbf{Results}

\textbf{SolA production in wild potato species}

To capture HF production across the Petota, RE was collected from 336 different accessions obtained from the CGN and including the cultivated potato accessions \textit{Solanum tuberosum} Bintje, MF-II and TPS-67 commonly used in potato research (Prakash et al. 2020). The REs were filtered and concentrated using Solid Phase Extraction (SPE) and analysed using UHPLC-MS/MS (Fig. 1a). The presence of SolA was confirmed with a standard that was injected at regular intervals during the analysis. In total, SolA was detected in 105 samples including in \textit{Solanum tuberosum} cv. Bintje, MF-II and TPS-76. A total of 231 accessions did not produce a detectable level of SolA (Suppl. Table S1).

We observed large variation between some of the species. For instance, a low amount of SolA was detected in RE of \textit{Solanum megistacrolobum} 699-1, while a high amount of SolA was detected in the RE of \textit{Solanum kurzianum} 276-3 (Fig. 1b). Also within species, between different accessions, there was a variation in SolA content, for instance SolA was only detectable in 8 out of 13 accessions of \textit{Solanum microdontum} var. gigantophyllum (Suppl. Table S1). SolA was not detected in any of the accessions of 29 species. For example, the REs of all 12 accessions of \textit{Solanum verrucosum} did not contain any detectable amount of SolA, just as the 15 accessions of \textit{Solanum bulbocastanum}. 
Fig. 1. (a) Summary of the pipeline for RE collection, sample preparation and SolA analysis. (b) Chromatograms obtained by UHPLC-MS operating in positive ionization mode \((m/z = 499)\) showing the presence of SolA in some of the analysed samples and the absence in others. The black line represents a standard of SolA (0.031 pmol/µl); the red line represents \textit{Solanum kurtzianum} 276-3 producing a high amount of SolA; the yellow line represents \textit{Solanum megistacrolobum} 699-1 producing a low amount of SolA; the green line represents \textit{Solanum pinnatisectum} 777-2 in which no SolA was detected. (c) Pie chart showing the proportions of the accessions screened: in red the number of species with a single accession that do not produce SolA, in blue the number of species with a single accession that produce SolA, in green the number of species with more than one accession where all do not produce SolA, in purple the number of species with more than one accession where all produce SolA and in orange the number of species with more than one accession where some of the accessions produce SolA and the rest do not produce SolA.
Based on the Amplified Fragment Length Polymorphism (AFLP) data of Jacobs et al. (2011), we performed a phylogenetic analysis of the Petota accessions, and decorated the tree to highlight SolA producer and non-producer accessions (Suppl. Fig. 1). SolA producers are present in most of the main clades, with few exceptions where all the accessions that do not produce SolA are grouped together in separate clades (for example S. bulbocastanum and S. verrucosum).

To better quantify the amount of SolA produced by some of the accessions and assess how that affects hatching, we selected 29 accessions that displayed a large array of SolA levels and regrew them with replicates. In addition to collecting the RE, also root weight was determined to allow for accurate quantification of the SolA production. As anticipated, all 29 accessions produced SolA, in concentrations ranging from 0.55 to 672 pmol/g fresh root weight (Suppl. Table S2).

**SolA producing accessions induce hatching of G. pallida**

This wide range of SolA concentrations presented a great opportunity to assess the relationship between SolA concentration and hatching rate and therefore a hatching experiment was performed using G. pallida D383. The hatching rate was over 50% for most of the accessions, but, surprisingly, there was no obvious correlation between SolA concentration and hatching (Fig. 2). The RE of accession MRL 79-04, the highest producer of SolA, induced just very low hatching, while the RE of accessions such as PNT775-1 and PNT375-1, containing the lowest amounts of SolA, induced 70-80% of hatching. These results suggest that the range of SolA concentrations we had is already saturating the hatching response or even supra-optimal in the highest producers, as already observed by Guerrieri et al. (2021) for tomato. There may also be, however, other HFs and/or hatching inhibitors in the RE of the Petota species that interfere with the relationship.
Fig. 2. Dot plot of hatching of *G. pallida* eggs treated with RE of SolA producing accessions. Line shows the linear relationship between SolA concentration and hatching.

**Accessions that do not produce SolA do also induce hatching**

The above results prompted us to also investigate the hatching inducing activity of the REs of accessions without SolA. Thus, a subset of 131 accessions from the 231 that did not produce SolA was used for hatching assays (Fig. 3).

Results from PERMANOVA analysis indicate that most of the variance within the data set was explained by differences between accessions (62%). Pairwise testing (Suppl. Table S3 of the accessions versus the tap water hatching treatment showed that REs from 11 accessions inhibited hatching while 40 accessions (including MF-II and KTZ 276-3) stimulated it. As illustrated in the right part of Fig. 3, a total of 10 accessions, despite not producing SolA, induced hatching similar as the positive controls KTZ 276-3 and MF-II (in gray in the figure). Accession PNT 777-2 (*S. pinnatisectum*) was the most effective with a hatching percentage of 73%. Indeed, most of the *S. pinnatisectum* accessions induced considerable hatching, with hatching percentages ranging from 43 to 73%. 
Fig. 3. Hatching of *Globodera pallida* D383 induced by 131 REs from Petota accessions. Box plots indicate the median of 5 replicates (black line). Grey bars represent (from left to right) the tap water control, accession KTZ 276-3 and accession MF-II, respectively. The hatching data are presented as percentages of the total amount of eggs per well. PERMANOVA showed there was a significant accession effect on hatching. Following the mass screening of the non-SolA producers, we regrew three of the strongest inducers of hatching (PNT 204-1, PAM 288-2 and PNT 777-2), to verify the absence of SolA. In addition, we regrew two SolA producer accessions to use them as controls (KTZ 273-6 and MF-II). The SolA concentrations in the REs were analysed and the absence of SolA was confirmed for both PNT accessions. Four out of five replicates of PAM 288-2 RE contained traces of SolA with signal over noise (S/N) ratio between 7 and 3 (Suppl. Table S4), levels that are almost 48-fold lower than in KTZ 273-6 and 198-fold lower than in MF-II, indicating that SolA is most likely not the main HF in the RE of PAM 288-2.
Other hatching factors in the root exudates of wild potato species specifically induce hatching of *Globodera pallida*

The high hatching in *G. pallida* D383 with non-SolA containing REs prompted us to test if these results are nematode species-specific by testing if they also induce hatching in *G. rostochiensis*. Hereto, the REs of two SolA producers (MRL 79-04 and ALB 461-3) with the highest SolA level around 150 pM, but inducing only 30-50% of hatching in *G. pallida* D383 (Fig. 2) and three accessions that did not produce SolA (PNT 204-1, PAM 288-2 and PNT 777-2), but inducing high hatching in *G. pallida* (Fig. 3) were selected for hatching experiments using *G. rostochiensis*. Strikingly, as shown in Fig. 4a, the non SolA producers did not induce significant hatching while they did induce hatching in *G. pallida*, suggesting that these accessions produce another hatching factor, which does induce hatching in *G. pallida* but not in *G. rostochiensis*. SolA producing accession ALB 461-3 induced around 40% hatching in *G. rostochiensis* just as in *G. pallida*. In contrast to ALB461-3, the hatching of *G. rostochiensis* induced by MRL 79-04 was rather low despite the high amount of SolA detected in the RE, this result hints at a possible presence of hatching inhibitors, specific for *G. rostochiensis* or at a higher sensitivity of this species to SolA.

To get more insight into the presence of other HFs in the accessions not producing SolA but still inducing hatching in *G. pallida*, we fractionated the REs of KTZ 273-6, MF-II, MRL 79-4, ALB 461-3, PNY 777-2, PAM 288-2 and PNT 204-1 and performed a hatching assay with *G. pallida* on the resulting fractions (Fig. 4b). As determined before (Guerrieri et al. 2021), SolA elutes in fraction 9. As shown in Fig. 4b, the hatching of *G. pallida* is highest in the first fractions where polar compounds elute for all the accessions tested. The hatching capacity of PAM 288-2 and PNT 204-1 in the first fraction is significantly higher than in the crude RE, indicating that there must be hatching factors present in the first fraction and hatching inhibitors in the crude RE. If we compare the hatching rate of the crude RE with fractions 8 and 9, even without detectable SolA, we can see that the hatching in these two fractions is higher than the crude RE (around 20 %), which could indicate the presence of others HFs in these fractions. Interestingly fractions 7 and 8 of PNT 777-2 induced hatching of 40-50%, similar to the crude RE. Since PNT 777-2 does not produce SolA, this result suggests that there are other HFs, likely similar to SolA, present in these fractions as also observed for PAM 288-2 and PNT 204-1. Since fraction 1 induces 20% hatching, it seems that the polar compounds present here are contributing together with the compounds in fractions 7 and 8 to the total hatching capacity of PNT 777-2 RE. Strangely fractions 8 and 9 of KTZ and MFII even if they contain SolA, do not induce high hatching compared to the crude RE. This is perhaps due to incomplete resuspension of the apolar compounds, such as SolA, in these fractions resulting in limited hatching activity.
Fig. 4 Hatching stimulation of PCN by the RE of selected *Petota* accessions. In a *G. rostochiensis* Mierenbos was exposed to non-fractionated RE of the same accessions. In b, *G. pallida* D383 was exposed to different HPLC fractions (described in the Material and Methods) of the RE of selected *Petota* accessions.
Discussion

SolA is so far the only, highly active HF for PCN, and has only been detected in potato and tomato (Schenk et al. 1999). Recently Guerrieri et al. (2021), showed that SolA is also produced by tomato relatives such as *S. pimpinellifolium*, *S. pennellii* and *S. habrochaites* and the trap crop *Solanum sisymbriifolium*. In the present work, for the first time, we demonstrate that SolA is also produced by wild relatives of potato belonging to the section *Petota*, suggesting that the relationship between SolA and the *S. tuberosum* species can be traced back to its center of origin in South America from where these species originate. We also showed that many accessions induce hatching even though they do not produce SolA, and fractionation suggests that there are other HFs produced by these accessions. We provide evidence that different PCN species display specificity for different HFs.

In this work, we showed that the production of SolA is not restricted to specific species in the *Petota* section. In our previous work (Guerrieri et al. 2021), we also proved the presence of SolA in the REs of wild tomato species belonging to the section *Lycopersicon* (Spooner et al. 2005), and also in *S. sisymbriifolium* which belong to a sister group of the section *Torva* (Miz et al. 2008). These species were all collected in South America and may have exchanged genetic material (Jacobs et al. 2011). Thus, the SolA production trait likely descended from a common ancestor of tomato and potato and their wild relatives. However, SolA production is not conserved throughout the phylogeny of the wild potato species (Suppl. Fig. 1), and we have observed intra- and inter-specific variation in the SolA level. Natural variation in secondary metabolite production has been studied in many cases e.g. in indole alkaloids, terpenoids and flavonoids. Variation in these metabolites has been attributed to genetic variation within or between species or to differences in the environment (e.g., herbivore presence, microbial communities and abiotic factors). From the biochemical point of view, variation in SolA levels in the RE between Petota species could be due to a change in SolA biosynthesis, as was shown for strigolactones in sorghum. Gobena et al. (2017) reported a mutation in the strigolactone biosynthetic gene *LOW GERMINATION STIMULANT 1* and the consequent loss of function resulted in a change in the strigolactone composition in sorghum RE. Subsequently, the main strigolactone produced was not 5-deoxystrigol, a highly active *Striga* germination stimulant, but orobancol which did not stimulate its germination, thus making the plant resistant.

The present study shows that there is no linear relationship between SolA level in the RE and hatching rate (Fig. 2), suggesting that the hatching rate is determined by a combination of HFs and hatching inhibitors. For example, as shown in Fig. 3, a high amount of SolA not always results in higher hatching. Accession MRL 79-04, for example, produced one of the highest concentrations of SolA, but induced just low hatching. This could be due to an inhibitory effect of very high concentrations of SolA on PCN hatching (Sakata et al. 2020). However, the presence of hatching inhibitors can also not be excluded, as demonstrated by Byrne et al. (1998). Indeed, according to our statistical analysis, 11
accessions showed inhibition towards PCN hatching (Suppl. Table 3). Also our fractionation results suggest the presence of inhibitors, for example in the RE of PAM 288-2 and PNT 204-1 for which the first fraction induced higher hatching than the crude RE (Fig. 5b).

Interestingly, several accessions that do not produce SolA induced high hatching, suggesting that SolA is not the only HF produced by wild potato relatives. Shimizu et al. (2020), showed that potato SGAs (α-chaconine and α-solanine) have a certain degree of hatching stimulation capacity in PCN, with solasonine showing the highest activity. Our fractionation results for some genotypes showed that the first fractions are quite active in inducing hatching. Based on the retention time, these fractions must contain polar compounds, possibly belonging to the SGAs.

The fractionation of the RE of the non-SolA producers PAM 288-2, PNT 204-1 and PNT 777-2 (Fig. 4b), showed considerable hatching activity present in some of the less polar fractions (fractions 7 and 8), to a similar level as the crude RE. These two fractions eluted slightly earlier than SolA, which elutes in fraction 9. This suggests that there is a compound(s) present in fractions 7 and 8 that is similar to SolA. This possibility is supported by the structural diversity in one of the other CN hatching factors, glycinoeclepin. Glycinoeclepin A was the first HF identified to be active towards the soybean cyst nematode (Masamune et al. 1982). In 1985, Fukuzawa et al., isolated two additional compounds with a structure similar to glycinoeclepin A, that were coined glycinoeclepin B and C. Both compounds were isolated using bio-assay guided fractionation, but while glycinoeclepin B induced hatching, albeit at a higher concentration than required for glycinoeclepin A, glycinoeclepin C showed no hatching activity.

So far we do not know what the structure is of the SolA-like compound for which we have indications it is present in the REs of some of the species we have investigated. We can also not exclude that there is more than one, just as in kidneybean. Identification of these putative new HFs, opens up the possibility to investigate PCN species-specificity of these compounds. We provide evidence that G. rostochiensis is less sensitive to this new HF(s) than G. pallida suggesting that there may be differences in how different PCN species respond to different Petota species.

Despite the high activity of SolA as HF, the lack of an analytical method prevented studies on its evolution and presence in wild potato varieties, making it impossible to consider SolA as a breeding trait. The present study is the first step towards the identification of species and accessions that can potentially be used as breeding materials for future commercial potato varieties that do not stimulate PCN hatching or, vice versa, the creation of new trap crops that induce high hatching of PCN, but are not a host or are harvested before the main crop is planted, thus reducing PCN infection (Scholte 2000). Our findings also suggest the presence of hatching inhibitors that could be used to inhibit hatching in the field. The isolation and identification of the putative SolA-like compounds we report here will be an important step towards further investigating their structural diversification in the Solanaceae, and co-evolution between SolA biosynthesis with PCN.
References


Oka Y (2020) From old-generation to next-generation nematicides. Agronomy 10:. https://doi.org/10.3390/AGRONOMY10091387


Supplemental materials

https://www.dropbox.com/sh/auw1tfa6lgi362p/AAA3eAv7t7rqO_6WqVFq4G2Sa?dl=0

**Supplemental Table S1:** Summary of the experiment with indication of the species and the accessions analysed, source code, country of origin and presence/absence of the species in the phylogenetic tree of Fig. 2. Presence or absence of SolA in the RE is indicated with a green or red label respectively.

**Supplemental Table S2:** Information about the samples collected from the 29 SolA producers species regrown: the name of the samples, the root mass expressed in grams of fresh weight (g FRW), the amount of SolA detected in the root exudates (RE) and the concentration of SolA in the final solution of *G. pallida* hatching assay. The amount of SolA is given as pmol per 200 ml of RE, pmol/ml of RE, pmol/g FRW, ng/g FRW and nM of RE.

**Supplemental Table S3:** Pairwise testing results of the 131 non-producers accession versus tap water hatching. The significance was calculated at 10, 12, 14, 16, 18 and 20 days during the hatching assay.

**Supplemental Table S4:** Information about the samples collected from non SolA producers PAM 288-2, PNT 204-1 and PNT 777-2 and controls KTZ 276-3 and MF-II regrown. Are indicated: name of the samples, number of replicates, peak area of SolA, signal over noise ratio (S/N), root mass expressed in grams of fresh weight (g FRW) and amount of SolA detected in the root exudates (RE). The amount of SolA is given as pmol per 500 ml of RE, pmol/g FRW and ng/g FRW.

**Supplemental Fig. S1** Neighbour joining tree of the collection of wild potato accessions analysed. The accessions in which SolA was detected are indicated in green, the species mentioned in the text as no producing SolA (*S. bulbocastanum* and *S. verrucosum*) are indicated in red.