Chapter 6.

Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are known as feeding deterrents and toxic compounds to generalist herbivores. Among the PAs of *Jacobaea vulgaris,* jacobine and erucifoline are the most effective against insect herbivores as indicated by correlative studies. So far, little is known on the effect of jacobine and erucifoline as individual PAs. We, therefore, isolated these PAs from their respective *Jacobaea* chemotypes. These and other commercially available senecionine-like PAs including, senecionine, seneciphylline, retrorsine, and senkirkine were tested as free base and *N*-oxide forms. A range of concentrations from 0–70 ppm was added to *Spodoptera exigua* cell lines and injected into the haemolymph of 3rd instar larvae. Both bioassays led to similar results in the order of PA toxicity, indicating that the cell lines are a valuable tool for a first toxicity screen. Testing individual PAs, jacobine and erucifoline appeared to be the most toxic PAs proving their major role in plant defence against generalist herbivores. Senkirkine and seneciphylline showed a lower toxicity than jacobine and erucifoline but higher than retrorsine. Senecionine was not toxic at the tested concentrations. In all toxic PAs the free base form was more toxic than the *N*-oxide form. Combination of toxic PAs with chlorogenic acid, another reported defence compound in *Jacobaea*, resulted in a reduction of PA toxicity. Our results stress that structural variation of PAs influences their effectiveness in plant defence.

Key Words- *Spodoptera exigua*, cell line bioassay, insect injection bioassay, pyrrolizidine alkaloids, toxicity.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a class of secondary plant metabolites well known for their negative effects on insect herbivores and vertebrates. In insect herbivores PAs can act as feeding deterrents and toxic compounds (Macel et al., 2005; Ober and Kaltenegger, 2009; van Dam et al., 1995). In vertebrates they can have hepatoxic, pulmotoxic (Mattocks, 1986; Cheeke, 1988) and carcinogenic effects (Frei et al., 1992).

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). In members of the *Jacobaea* genus (Asteraceae), PAs exist as macrocyclic diesters with two types of necine bases: retronecine and otonecine (Fig. 1). So far over thirty five PAs have been reported in *Jacobaea* plants (Cheng et al., 2011; Pelser et al., 2005). Based on the biosynthetic route, these alkaloids are divided into four main groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). Most of the PAs occur in two interchangeable forms: free base (tertiary amine) and *N*-oxide (Hartmann and Dierich, 1998). So far the *N*-oxide is accepted as the major storage form in plants (Hartmann and Toppel, 1987; van Dam et al., 1995) and generally the concentration of this form is higher than that of the related free base (Joosten et al. 2011). The free base form is considered to be more toxic than the *N*-oxide one (van Dam et al., 1995; Hartmann, 2007).

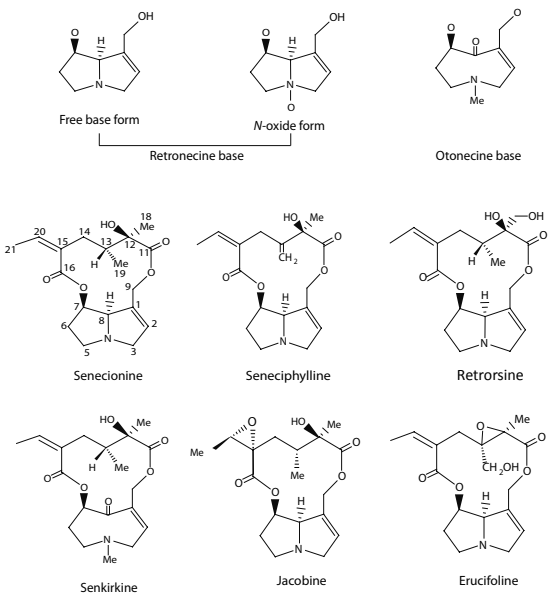

Fig 1. Chemical structures of pyrrolizidine alkaloids tested in insect cell line and injection bioassays.

When ingested by vertebrates the *N*-oxide form is not toxic but when entering the gut the *N*-oxide is reduced to its free base, which is then converted by cytochrome P450 enzymes (CYPs) into unstable pyrrole intermediates in the liver. These intermediates readily react with the amino groups of proteins as well as with nucleosides in DNA and RNA (Wiedenfeld and Edgar, 2011).

Similarly, in insects, PAs are reduced in the gut and converted by CYPs to the highly reactive pyrrole intermediates (Lindigkeit et al., 1997). Besides these, the potential of PAs as neurotoxins has been demonstrated. The free base form of PAs showed a significant binding activity to membranes of muscarinic acetylcholine and serotonine receptors derived from porcine brain, which may influence neuronal signal transduction as well as central nervous system- and muscular- activity (Schmeller et al., 1997).

As such structural variation of PAs may influence their effectiveness in plant defence. Several *in-vivo* plant studies have addressed this question. Especially jacobine- (Leiss et al., 2009a; Cheng et al., 2011; Joosten, 2012) and erucifoline like PAs (Macel, 2003; Macel and Klinkhamer, 2010) were identified to contribute to plant defence. However, these results are mainly based on correlative studies. Little is known on the effect of jacobine and erucifoline as individual PAs. There is only one report on erucifoline, isolated from the Canarian endemic plant *Canariothamnus palmensis*, demonstrating a negative effect on green peach aphid *Myzus persicae* (Dominguez et al., 2008). Mainly senecionine-like PAs have been individually tested (Lindigkeit et al., 1997; Macel et al., 2005) since these are the only PAs commercially available. However, in contrast to senecionine-, jacobine and erucifoline like PA are mainly involved in plant defence against generalist insects. In the current study we, therefore, isolated jacobine and erucifoline from their respective *Jacobaea* chemotypes for application in lepidopteran insect bioassays.

Most studies on insect toxicity use feeding bioassays in which insects are presented with artificial diets or leaf surfaces to which the compounds to be tested have been added. However, this approach is often tedious, time consuming and the amounts of PAs eaten by the insects may vary. Moreover, such assays need relatively large amounts of the test compounds (Decombel et al., 2004). The latter is especially problematic for PAs, of which only a few are commercially available and which, therefore, need to be isolated first in order to test them. For insect toxicity bioassays the amount of compound needed can be greatly reduced by using injection bioassays in which the compound is directly injected into the haemolymph. However, for routine screening purposes this method remains too laborious. Using insect cell lines as a bioassay system appears to form an interesting alternative to test PA toxicity (Dinan et al., 1990). Cell based bioassays are commonly used in life sciences to study biological activities, toxicity and cellular processes. Cell lines of the beet armyworm, *Spodoptera exigua* (family Noctuidae; order Lepidoptera), have been successfully used for screening insecticide activities (Decombel et al., 2004). In the present study we, therefore, compared the toxicity of individual PAs in, *S. exigua* cell lines with that of an insect injection bioassays.

In this study, we specifically addressed the following questions:

1. Do different types of PAs have different toxic effects on *S. exigua*?
2. Do the free base and *N*-oxide forms of PAs have different toxic effects on *S. exigua*?
3. Next to PAs chlorogenic acid (CGA) is a common defence compound occurring in *J. vulgaris* (Nuringtyas et al, 2012, Leiss et al., 2009). Does the combination of PAs and CGA lead to additive or synergistic effects of toxicity to *S. exigua*?
4. Is the toxicity of PAs to *S. exigua* measured in cell lines comparable to that measured in an injection bioassay?

METHODS AND MATERIALS

Pyrrolizidine Alkaloids. We tested crude PA extracts containing a mixture of PAs present in the *Jacobaea* plants as well as individual PAs including senecionine-like PAs: senecionine, seneciphylline, senkirkine (Carl Roth, Karlsruhe, Germany), retrorsine and retrorsine *N*-oxide (Sigma, St. Louis, USA) as well as jacobine and erucifoline (Fig. 1). Jacobine and erucifoline were isolated from the respective chemotypes of *J. vulgaris*. For PA extraction, a modified procedure of Hartmann and Toppel (1987) was used, in which 0.05 M H_2SO_4 was replaced with 3% formic acid. Since the extraction of the erucifoline chemotype resulted in a low amount of extract, this extract was used only for isolation of erucifoline. The larger amount of extract obtained from the jacobine chemotype was used for isolation of jacobine as well as for preliminary toxicity bioassays to define the range of PAs concentrations to be tested. Isolation of jacobine and erucifoline was carried out using Centrifugal Partition Chromatography (CPC) and HPLC (Hartmann and Zimmer 1986; Hösch et al. 1996). Except for senkirkine all the PAs tested occur in plants as both *N*-oxide or free base form. To obtain the *N*-oxide form the PA extract as well as the corresponding free base, individual PAs were oxidised with *m*-chloroperbenzoic acid and purified using column chromatography with an alkaline alumina gel (Craig and Purushothaman 1970) except for retrorsine *N*-oxide which was available commercially. The result of the *N*-oxidation process was confirmed by Thin Layer Chromatography (TLC) and ^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR).

Spodoptera exigua Cell Culture Bioassay.
Pyrrolizidine alkaloid extracts. PA extracts were dissolved directly in DMSO. For a first preliminary experiment, we used a concentration range of 0 – 220 ppm. The range of 0 –70 ppm appeared to be most effective and was thus used in the later experiments.

Individual PAs. Each individual PA was dissolved directly in DMSO. Seven different concentrations in the range of 0 – 70 ppm were used.

Individual PAs combined with CGA. Individual PAs and CGA, both dissolved in DMSO, were combined. Individual PAs, which proved to be toxic and senecionine as a representative of a non-toxic PA were included in this bioassay. Seven concentrations of PAs ranging from 0–70 ppm were used to which 45 ppm CGA, which is representative for the amount of CGA in plants (Leiss et al., 2009b) was added. In addition, seven concentrations of CGA in the range of 0–70 ppm were tested.

Cell Lines. A sample of *S. exigua* cell line SE301 (Hara et al., 1995) was obtained from a cell culture routinely grown at the Laboratory of Virology, Wageningen University, The Netherlands. These cells were originally derived from neonate larvae. Cell lines were propagated at 27 °C in a Hyclone CCM3 medium (Thermo Scientific, Utah, USA) enriched with 5% Fetal Bovine Serum (FBS; Gibco, Auckland, NZ). To prevent contamination during the experiments, antibiotic gentamycine (Sigma, St. Louis, MO, USA) was added to the medium at 50 µg/ml. The cell line was sub-cultured every four days in 25 cm^2 tissue culture flasks (Corning Inc, New York City, USA).

Cell Culture Bioassay. The PA extract and individual PAs in both *N*-oxide and free base form were tested in a dose-response experiment. Two kinds of controls were applied, a negative control

consisting of 2 µl DMSO and a positive control consisting of 2 µl of 5 ppm Abamectine (Sigma, Chemical, St. Louis, USA), which is equivalent to the concentration inhibiting cell growth by 50% (IC_{50}). Abamectine is the active compound of the commercial insecticide Agri-Mek (Syngenta). Cells were collected two days after sub-culturing when they were in the exponential growth phase. Cells were diluted with fresh medium without serum to a density of 10^5 cells/ml. After loading each well of a 96-well culture plate (Costar, Corning Inc, New York, USA) with 200 µl of the cell solution (2.10^4 cells), 2 µl of compound solution to be tested was added with a micropipette (Gilson, USA). For each concentration tested nine replicates were used. Each compound was applied in threefold using three different plates. The compounds to be tested were randomly applied to each plate. After incubation for two days at 28ºC and 90% humidity, the cell numbers per cultured well were counted with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell-counting technique, described below. The IC_{50} concentrations were calculated using probit analysis.

MTT Assay. MTT (Sigma) solution was prepared as a 5 mg/ml stock in fetal bovine serum (FBS; Gibco, Auckland, NZ, USA). This solution was sterilised by filtration through a 0.2 mm filter (Acrodisc, Pall Co., Ann Arbor, MI, USA) and stored at 2–8°C. Twenty µl MTT solution was added to each well and incubated at 37°C for 3 h. At the end of the incubation period, the medium was removed carefully without disturbing the cells. The converted dye was solubilised with 200 µl acidic isopropanol (0.04 M HCl in absolute isopropanol). Subsequently, the optical densities (OD) were recorded at 575 nm in an automatic 96-well microtiter plate reader (Sigma Aldrich). In initial tests, we established a good linear relation between OD and cell numbers of the *S. exigua* cell cultures (Fig. S.1).

Spodoptera exigua Injection Bioassay.
Pyrrolizidine alkaloid preparation. An initial test with 2% DMSO resulted in 50 % larval mortality. Therefore, individual PAs were dissolved in 50% ethanol. We selected a number of PAs to represent PAs with the highest, intermediate and the lowest IC_{50} values based on the cell line bioassay: jacobine, retrorsine, seneciphylline and senecionine. Since the highest toxicity observed corresponded to an IC_{50} of 35 ppm we used three concentrations in the range of 0 – 50 ppm for injection which equals 0 – 50 µg/larva.

Insects. Third instars caterpillars of *S. exigua* were obtained from a laboratory culture reared on an artificial diet (Singh 1983) in a growth chamber at 30ºC, 16h/8h L/D photoperiod, 70% RH. The larvae used were 100–120 µg in weight.

Injection bioassay. One microlitre of individual PAs was injected into the haemolymph of a third instar larvae of *S. exigua.* For each concentration twenty four larvae were used. Injections were performed under a binocular microscope using a microsyringe (SGE GC, Australia) which was inserted into the 5^{th} larval segment. During injection, the larvae were held immobile with a thin metal scalpel. After injection, the larvae were kept together on an artificial diet in a plastic container (diameter 8.5 cm, height 8 cm) covered with tissue paper for proper aeration. Containers were randomly placed in a growth chamber at 27ºC, 16h/8h L/D photoperiod, 70% RH. After 24h, larval mortality was counted. A larva was considered dead when no response to the touch

of a glass rod was observed. Two kinds of controls were used. First, an empty injection, and second an injection with the solvent only. Each bioassay was conducted twice. The number of surviving larvae of the treatment was compared with that of the solvent control using a Chi-square test. Furthermore, the LC_{50}, the concentration causing 50% larval mortality, was calculated using probit analysis. The correlation between the two injection bioassays was analysed with a Pearson correlation analysis. All statistic analyses were performed using IBM SPSS statistics 19 (Chicago, IL, USA).

RESULTS

Cell Culture Bioassay.

PA extracts. In the initial experiments with concentrations ranging from 0–220 ppm, 90 % of the larvae died at concentrations of 90 ppm and higher (Fig. 2a). Therefore, we narrowed the concentration range of the PA extract to 0-70 ppm. The free base form with an IC_{50} of 30.03 ppm showed a higher toxicity compared to the *N*-oxide form with an IC_{50} of 50.24 ppm (Fig. 2b).

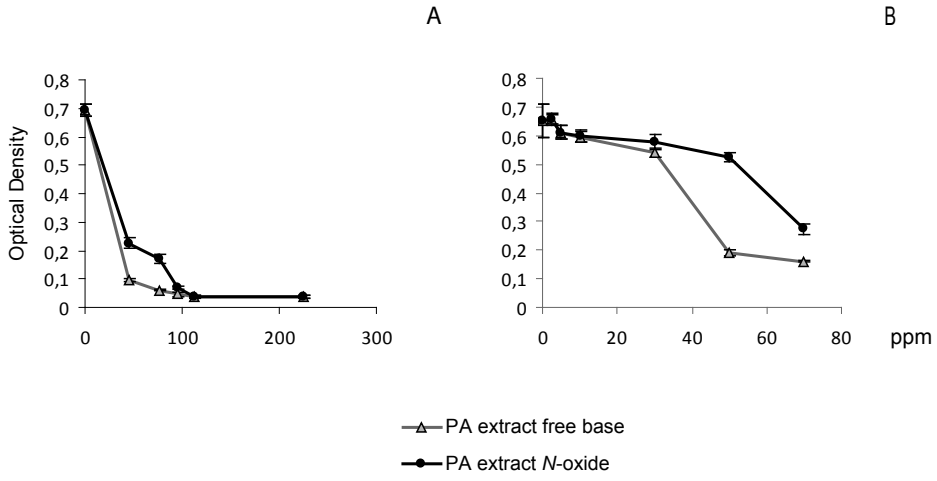

Fig 2. The bioactivity of PA extracts as free base and *N-oxide* form in *Spodoptera exigua* cell lines at concentrations of 0-220 ppm (A) and 0-70 ppm (B), measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated

Individual PAs. All *N*-oxide forms tested had an IC_{50} value > 70 and we considered them, therefore, as non- toxic at the tested doses (Fig. 3). Except for senecionine and retrorsine all free-base forms, were toxic, jacobine showed the highest toxicity with an IC_{50} of 34.95 ppm followed by erucifoline at 36.92 ppm (Fig. 3). The senecionine-like PAs senkirkine and seneciphylline showed an IC_{50} value of 43.15 and 47.75 ppm, respectively.

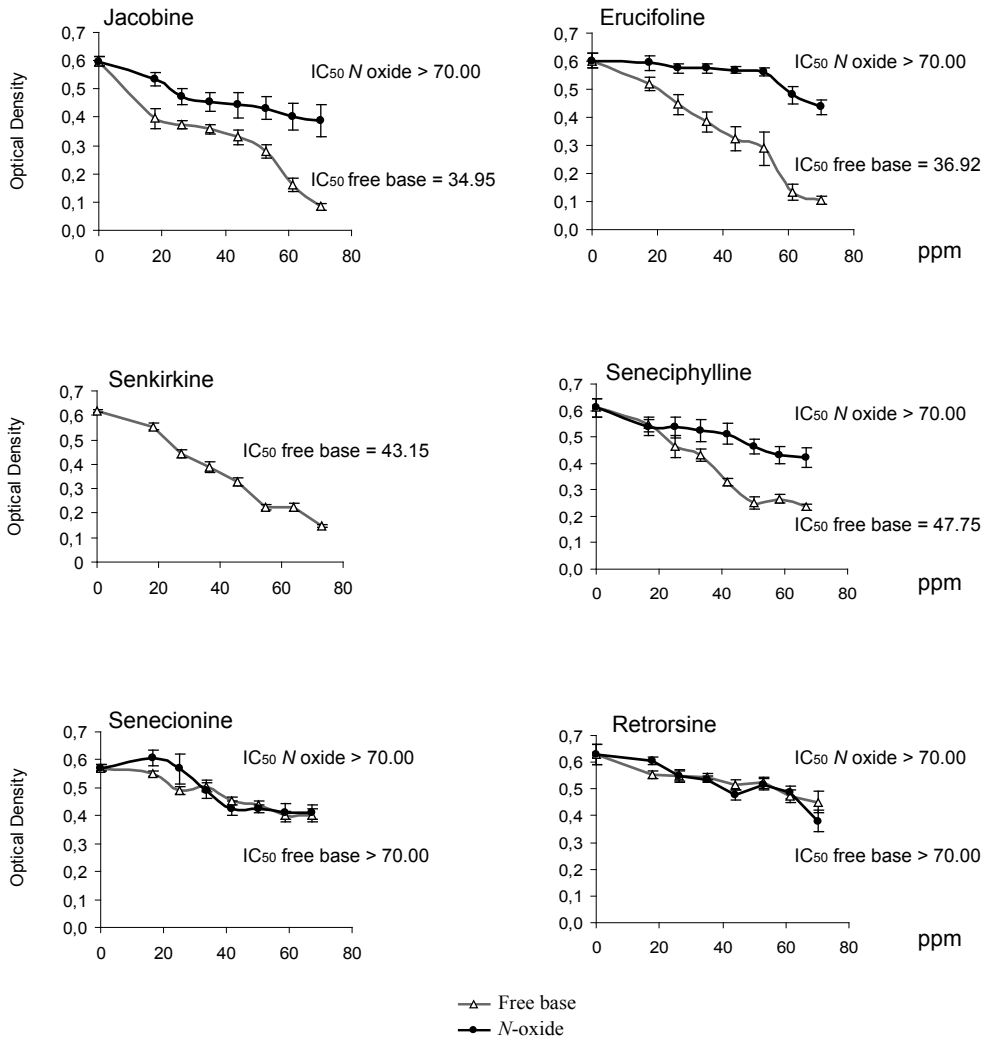

Fig 3. The bioactivity of individual PAs as free base and *N-oxide* form in *Spodoptera exigua* cell line at concentrations of 0-70 ppm measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated. The IC_{50} is equivalent to the concentration inhibiting cell growth by 50%

Individual PAs combined with CGA. Chlorogenic acid showed an IC_{50} of 67.70 ppm (Fig. 4). In general, the combination of CGA at 45 ppm that is IC_{24} and PAs resulted in a reduction of PA toxicity as expressed by the lower IC_{50} values of the mixture compared to the respective individual PAs (Fig. 4). The strongest reduction of toxicity was observed in a mixture with erucifoline amounting to an IC_{50} >70 ppm, indicating that erucifoline toxicity was highly reduced. Similarly, the IC_{50} of jacobine and seneciphylline increased when CGA was added. Senecionine did not show any toxic effect either as individual PA or in combination with CGA.

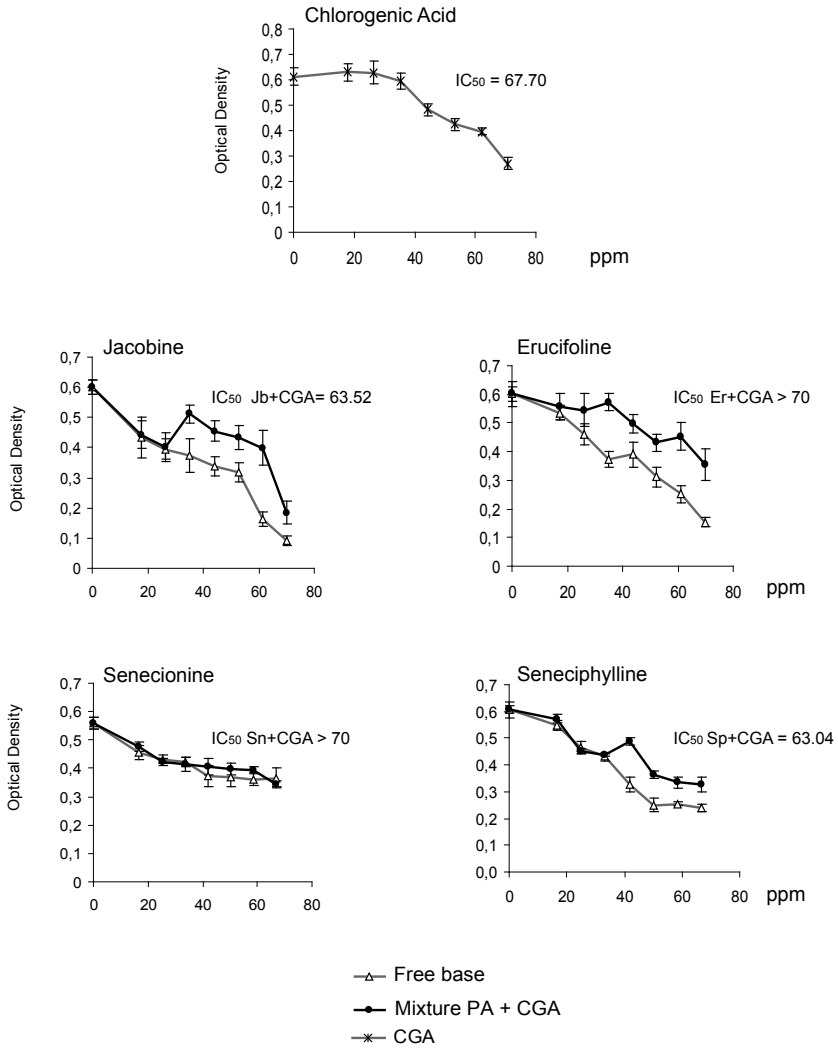

Fig 4. The effect of 45 ppm chlorogenic acid on the toxicity of individual PAs in *Spodoptera exigua* cell lines at concentrations of 0-70 ppm measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated. The IC_{50} is equivalent to the concentration inhibiting cell growth by 50%

Injection bioassay.

Except for seneciphylline all *N*-oxide alkaloids showed lower toxicity than the respective free base form (Fig. 5). The free base form of jacobine with an LC_{50} of 22.9 ppm was most toxic followed by seneciphylline and retrorsine an LC_{50} of 32.35 and 43.16 ppm, respectively. At the concentrations tested, senecionine as free base form was also not toxic. The bioassay was conducted twice and the results of both bioassays were significantly correlated for each PA tested (data not presented). In contrast to the free base form, the *N*-oxide form of the PAs showed no biological relevant toxicity in all PAs except for a low toxicity of the *N*-oxide. The result of the two experiments showed a

similar response as presented in Fig. 5. The free base form of jacobine was most toxic followed by seneciphylline and retrorsine, respectively. Senecionine as free base was also not very toxic at the doses tested.

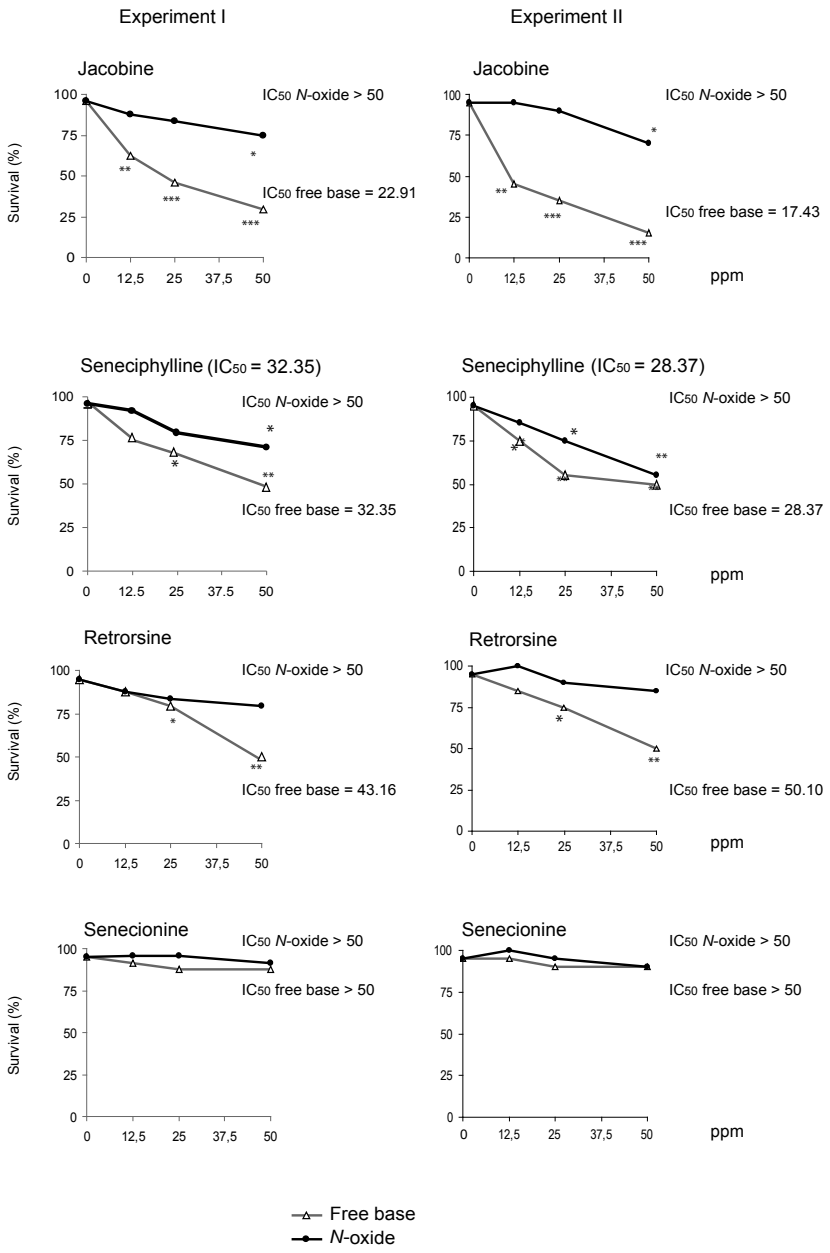

Fig 5. Survival of third instar larvae of *Spodoptera exigua* upon haemolymph injection with individual PAs at concentrations of 0-50 ppm. Data present the percentage survival from 24 larvae for each compound and concentration tested and the standard error. Significant differences between treatments and control are indicated as *** = P< 0.0001, ** = P < 0.001, * = P<0.05. The LC_{50} is equivalent to the concentration which caused death by 50% of the population.

DISCUSSION

Comparing the toxicity of PAs to the generalist herbivore *S. exigua* using cell line and injection bioassays, we observed in both assays similar results for the order of PAs toxicity. Jacobine was the most toxic, followed by erucifoline, senkirkine and seneciphylline, while senecionine was not toxic at the tested concentrations. Decombel et al. (2004) showed that the cytochrome P450 (CYPs) responsible for the formation of the highly reactive pyrrole intermediates were present in both *S. exigua* cell lines as well as the *in-vivo* insects. Therefore, most likely similar toxicity mechanisms are present in the two different systems, indicating that *S. exigua* cell lines are a valuable tool for a first screen of PA toxicity.

Our results confirm the prominent role of jacobine and erucifoline-like PAs in plant defence against leaf feeding insects. In *in-vivo* plant studies mainly the jacobine-like PAs were observed to be correlated to the above ground defence of *Jacobaea* plants against the generalist herbivore western flower thrips, *Frankliniella occidentalis*, including, jaconine (Joosten, 2012), jacobine *N*-oxide and jaconine *N*-oxide (Cheng et al., 2011; Joosten, 2012; Leiss et al., 2009a) as well as jacoline *N*-oxide (Cheng et al., 2011). Erucifoline, isolated from an extract of *C. palmensis*, demonstrated a negative effect on the green peach aphid (Dominguez et al.,, 2008). Comparing jacobine and erucifoline chemotypes in the laboratory Macel (2003) observed a negative effect of the jacobine chemotypes on *S. exigua*, *F. occidentalis*, and *M. persicae*, while the erucifoline chemotype affected the cabbage moth, *Mamestra brassicae*. Comparing the same chemotypes in the field, the erucifoline chemotypes showed less damage compared to the jacobine ones (Macel and Klinkhamer, 2010). This result was explained by the presence of the specialist cinnabar moth, *Tyria jacobaea*, which uses jacobine as an oviposition cue for host finding (Cheng et al., 2013).

Both, jacobine and erucifoline, the most toxic PAs in our study are retronecine macrocyclic diesters with epoxide functional groups. These functional groups may provide suitable sites for chemical modification by CYPs resulting in the formation of toxic pyrroles (Wiedenfeld and Edgar 2011). Furthermore, the presence of epoxide functional groups may inhibit detoxification by esterases (Culvenor et al. 1976). The ester bonds of PAs can be hydrolysed by esterases to form non-toxic necine and necid acid (Wiedenfeld and Edgar 2011). The rate of hydrolysis depends on the level of steric hindrance of the ester linkages. The more complex the PAs the higher the level of steric hindrance. Jacobine and erucifoline are both relatively complex branched structures, which may inhibit hydrolysis.

Senkirkine and seneciphylline in our study showed lower toxicity than jacobine and erucifoline. Senkirkine has been reported to significantly reduce the survival of western flower thrips at a concentration present in plants (Macel et al., 2005) and to deter sixth instar larvae of the spruce budworm, *Choristoneura fumiferana* (Bentley et al., 1984). Senkirkine, compared to senecionine was more genotoxic to the fruit fly, *Drosophila melanogaster* (Frei et al., 1992). Seneciphylline has been observed to be a feeding deterrent to the pea aphid, *Acrythosiphon pisum* (Dreyer et al., 1985) and the crane fly, *Cyllindrotoma distinctissima* (Hagele and Rowell-Rahier, 2000). The migratory locust, *Locusta migratoria* was more strongly deterred by seneciphylline compared to other senecionine-like PAs and was toxic to *M. persicae* (Macel et al., 2005). The necic acid

moiety of senkirkine is identical to that of senecionine, but contains an otonecine base instead of retronecine. In contrast to senecionine, it is not easily oxidised to an *N*-oxide. That means that fast excretion in the form of a more water soluble *N*-oxide is hindered and possibly explains the higher toxicity of senkirkine compared to senecionine. Seneciphylline contains a retronecine base, as does senecionine which is not toxic, but contains a methylene group at C-13 of the necic acid. Addition of a methylene group at the lactone ring of eremophilanes increased antifeedant and insecticidal activity against *S. littoralis* (Tan et al., 1998).

Senecionine was not toxic at the tested concentrations for both cell lines and *S. exigua* larvae. Macel et al. (2005) also did not observe deterrent feeding activity of senecionine in *S. exigua* larvae even at three times the plant concentration of 1.5 mg/g fresh weight. Dominguez et al. (2008) studying the effect of senecionine on *S. littoralis* did not report any effect either. However, senecionine has been reported to significantly reduce larval survival of *M. persicae* and to deter feeding of *L. migratoria* (Macel et al., 2005). Senecionine is the backbone of all PAs, thus it represents the simplest structure of the macrocyclic PAs, which might explain its non-toxic nature. It may be easily detoxified by hydrolysis and rapidly removed from the body. Indeed, larvae of *Spodoptera littoralis*, injected with 3.5 µg senecionine *N*-oxide were able to completely eliminate this PA within 24 hours (Lindigkeit et al., 1997). The rapid excretion of senecionine avoids its conversion into the toxic pyrrole. Excretion of senecionine by *S. exigua* may explain why this PA was not toxic in our study in contrast to its toxic effect on aphids and locusts. Our results showed that both retronecine- and otosenine based PAs were toxic to *S. exigua*. However, within the retronecine based PAs toxicity depended on the structure of the necic acid.Supporting the important of necic acid, Dreyer et al. (1985) after removing the necic acid of riddelliine, a senecionine-like PA, demonstrated that the remaining retronecine base was 100 times less toxic to *A. pisum* compared to the complete macrocyclic riddelliine.

The cell line and injection bioassay led to comparable results except for retrorsine, which showed toxicity in the insect but not in the cell line bioassay. Retrorsine is a monohydroxylated derivative of senecionine. The hydroxylation may increase the water solubility of the PA possibly facilitating excretion. Injection of retrorsine into the haemolymph may lead to high concentration; thus retrorsine may immediately reach essential organs before effective metabolism and excretion occur.

Both bioassays, the cell line and the injection bioassay, indicated that the free base form of the PAs was more toxic than the *N*-oxide form. Similarly, for the deterrent activity, *S. exigua* larva clearly preferred the *N*-oxide form of a *Cynoglossum officinale* PA extract to the free base form (van Dam et al., 1995). Also for other generalist insects such as *A. pisum* (Dreyer et al., 1985), *F. occidentalis*, *L. migratoria* (Macel et al., 2005) and *M. persicae* (Reina et al., 2001) the free base form was the deterrent one. The free base form was reported to show a significant binding activity to muscarinic acetylcholine and serotonine receptors (Schmeller et al., 1997). This might cause short-term physiological disturbance contributing to mortality. Further studies on the mechanisms of toxicity are required to be able to better understand the role of PAs in plant defence.

Chlorogenic acid was moderately toxic to the *S. exigua* cell lines. Feeding deterrence of CGA to *S.*

exigua larvae has been observed by Felton et al. (1989, 1991). A negative effect on various other caterpillars was observed *in-vitro* (Bernays et al. 2002) and *in-vivo* (Elliger et al., 1981; Huang and Renwick, 1995; Mallikarjuna et al., 2004). A combination of PAs and CGA decreased the toxicity of PAs in our experiments and in the case of erucifoline toxicity was lost completely. The negative effect of CGA on caterpillars is based on its prooxidant effect as has been demonstrated for *S. exigua* (Felton et al., 1989, 1991). CGA is readily oxidised to chlorogenoquinone which binds to proteins, free amino and nucleic acids. As such CGA possibly competes with the PAs for enzymatic oxidation. Furthermore, the unstable pyrroles formed through PA reduction may compete with chlorogenoquinone for binding to amino and nucleic acids. CGA in the leaves of *Jacobaea* plants was highly accumulated in the epidermis in contrast to the PAs, which were concentrated in the mesophyll (Nuringtyas et al. 2012). Based on our results, such tissue specific distribution would prevent the antagonistic activity of CGA on PA toxicity.

CONCLUSION

The comparison of PA toxicity to the generalist herbivore *S. exigua* using cell line and injection bioassays, led to similar results in the order of PA toxicity, indicating that the insect cell lines are a valuable tool for a first toxicity screening. Testing individual PAs, jacobine and erucifoline appeared to be the most toxic PAs proving their major role in plant defence against generalist herbivores, which so far has only been shown in correlation studies. As such our study shows that structural variation of PAs influences their effectiveness in plant defence. For a better understanding of toxicity, metabolism studies in cell cultures will be the next step in unravelling the biological activity of PAs.

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REFERENCES

Bentley, M., Leonard, D., Stoddard, W. and Zalkow, L. (1984) Pyrrolizidine alkaloids as larval feeding deterrents for spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Ann Entomol Soc Am* **77**, 393-397

Bernays, E.B., Chapman, R.C., and Hartmann, T.H. (2002) A highly sensitive taste receptor cell for pyrrolizidine alkaloids in the lateral galeal sensillum of a polyphagous caterpillar, *Estigmene acraea*. *J Comp Physiol A* **188**, 715-723

Cheng, D., Kirk, H., Mulder, P.P.J., Vrieling, K. and Klinkhamer, P.G.L. (2011) The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. *J Chem Ecol* **37**, 1071-1080

Craig, J. andPurushothaman, K (1970) Improved preparation of tertiary amine N-oxides. *J Org Chem* **35**, 1721-1722

Culvenor, C., Edgar, J., Jago, M., Outteridge, A., Peterson, J., and Smith, L. (1976) Hepato-and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. *Chem Biol Interact* **12**, 299-324

Decombel, L., Smagghe, G, and Tirry, L. (2004) Action of major insecticide groups on insect cell lines of the beet armyworm, *Spodoptera exigua*, compared with larvicidal toxicity. *In Vitro Cell Dev-An* **40**, 43-51

Dinan, L., Spindler-Barth, M. and Spindler, K.D. (1990) Insect cell lines as tools for studying ecdysteroid action. *Invertebr Reprod Dev* **18**, 43-53

Domínguez, D.M., Reina, M., Santos-Guerra, A., Santana, O., Agulló, T., López-Balboa, C. and Gonzalez-Coloma, A. (2008) Pyrrolizidine alkaloids from Canarian endemic plants and their biological effects. *Biochem Syst Ecol* **36**, 153-166

Dreyer, D.L., Jones, K.C. and Molyneux, R.J. (1985) Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J Chem Ecol* **11**, 1045-1051

Elliger, C.A., Wong, Y., Chan, B.G. and Waiss, A.C,, Jr. (1981) Growth inhibitors in tomato (*Lycopersicon*) to tomato fruitworm (*Heliothis zea*). *J Chem Ecol* **7**, 753-758

Felton, G.W., Doanto, K.K., Broadway, R.M. and Duggey. S.S. (1991) Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, *Spodoptera exigua. J Insect Physiol* **38**, 277-285

Felton, G.W., Donato, K., del Vecchio, R.J. and Duffey, S.S. (1989) Activation of plant foliar oxidases by insect feeding reduced nutritive quality of foliage for noctuid herbivores. *J Chem Ecol* **15**, 2667-2694

Frei, H., Lüthy, J., Brauchli, J., Zweifel, U., Würgler, F.E. and Schlatter, C, (1992) Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. *Chem-Biol Interact* **83**, 1-22

Hagele, B. and Rowell-Rahier, M. (2000) Choice, performance and heritability of performance of specialist and generalist insect herbivores towards cacalol and seneciphylline, two allelochemicals of *Adenostyles alpina* (Asteraceae). *J Evol Biol* **13**, 131-142

Hara, K., Funakoshi, M. and Kawarabata, T. (1995) A cloned cell line of *Spodopteta exigua* has a highly increased susceptibility to the *Spodoptera exigua* nuclear polyhedrosis virus. *Can J Microbiol* **41**, 1111-1116

Hartmann, T. (1999) Chemical ecology of pyrrolizidine alkaloids. *Planta* **207**, 483-495

Hartmann, T. (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. *Phytochemistry* **68**, 2831-2846

Hartmann, T. and Dierich, B. (1998). Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: biological need or coincidence? *Planta,* **206,** 443-451.

Hartmann, T. and Toppel, G. (1987) Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. *Phytochemistry* **26**, 1639-1643

Hartmann, T. and Zimmer, M. (1986). Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual *Senecio* species. *J Plant Physiol,* **122,** 67-80.

Hösch, G., Wiedenfeld, H., Dingermann, T. and Röder, E. (1996) A New High Performance Liquid Chromatography Method for the Simultaneous Quantitative Analysis of Pyrrolizidine Alkaloids and their *N*-Oxides in Plant Material. *Phytochem Anal* **7**, 284-288

Huang, X.P. and Renwick, J.A.A. (1995) Chemical and experiential basis for rejection of *Tropaeolum majus* by *Pieris rapae* larvae. *J Chem Ecol* **21**, 1601-1617

Joosten, L. (2012). Pyrrolizidine alkaloid composition of the plant and its interaction with the soil microbial community. In: *Plant Ecology and Phytochemistry, Institute Biology of Leiden*, PhD Thesis, Leiden Universtiy, Leiden.

Joosten, L., Cheng, D. D., Mulder, P. P. J., Vrieling, K., van Veen, J. A. and Klinkhamer, P. G. L. (2011). The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry,* **72,** 214-222.

Leiss, K. A., Choi, Y.H, Abdel-Farid, I., Verpoorte, R. and Klinkhamer, P.G.L. (2009a). NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. *J Chem Ecol,* **35,** 219-229.

Leiss, K. A., Maltese, F., Choi, Y. H., Verpoorte, R. and Klinkhamer, P. G.L. (2009b). Identification of chlorogenic acid as a resistance factor for thrips in *Chrysanthemum*. *Plant Physiol* **150,** 1567–1575.

Lindigkeit, R., Biller, A., Buch, M., Schiebel, H., Boppré, M. and Hartmann, T. (1997). The two faces of pyrrolizidine alkaloids: the role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. *Eur J Biochem,* **245,** 626-636.

Macel, M. (2003). On the evolution of the diversity of pyrrolizidine alklaoids: the role of insects as selective forces, PhD thesis, Leiden University, The Netherlands

Macel, M., Bruinsma, M., Dijkstra, S., Ooijendijk, T., Niemeyer, H. and Klinkhamer, P.G.L. (2005). Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *J. Chem. Ecol.,* **31,** 1493-1508

Macel, M. and Klinkhamer, P. G. L. (2010). Chemotype of *Senecio jacobaea* affects damage by pathogens and insect herbivores in the field. *Evol Ecol,* **24,** 237-250.

Mallikarjuna, N., Kranthi, K., Jadhav, D., Kranthi, S. and Chandra, S. (2004) Influence of foliar chemical compounds on the development of *Spodoptera litura* (Fab.) in interspecific derivatives of groundnut. *J Appl Entomol* **128**, 321-328

Ober, D. and Kaltenegger, E. (2009). Pyrrolizidine alkaloid biosynthesis, evolution of a pathway in plant secondary metabolism. *Phytochemistry,* **70,** 1687-1695.

Pelser, P., de Vos, H., Theuring, C., Beuerle, T., Vrieling, K. and Hartmann, T. (2005). Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae). *Phytochemistry,* **66,** 1285-1295

Reina, M., González-Coloma, A., Gutiérrez, C., Cabrera, R., Rodríguez, M.L., Fajardo, V. and Villarroel, L. (2001) Defensive chemistry of *Senecio miser*. *J Nat Prod* **64**, 6-11

Schmeller, T., El-Shazly, A. and Wink, M. (1997) Allelochemical activities of pyrrolizidine alkaloids: interactions with neuroreceptors and acetylcholine related enzymes. *J Chem Ecol* **23**, 399-416

Singh, P. (1983) A general purpose laboratory diet mixture for rearing insects. Insect Scie App **4**

Tan, R., Zheng, W. and Tang, H. (1998) Biologically active substances from the genus *Artemisia*. *Planta Med* **64**, 295-302

van Dam, N., Vuister, L., Bergshoeff, C., de Vos, H. and van Der Meijden, E. D. (1995). The "Raison D'être" of pyrrolizidine alkaloids *Cynoglossum officinale* :Deterrent effects against generalist herbivores. *J. Chem. Ecol.,* **21,** 507-523.

Wiedenfeld, H. and Edgar, J. (2011). Toxicity of pyrrolizidine alkaloids to humans and ruminants. *Phytochemistry Rev,* **10,** 137-151

Supplementary data Chapter 6

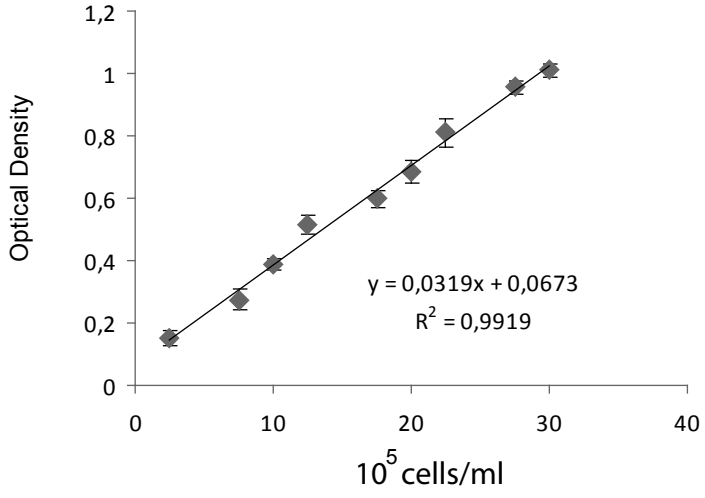

Fig S1. Standard curve for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a *Spodoptera exigua* cell line measured as optical density at 575 nm. Data present the average of nine replicates with the corresponding standard errors are indicated.