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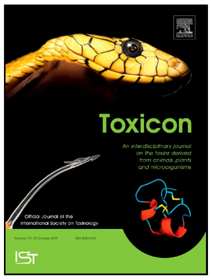

Senecio spp. transboundary introduction and expansion affecting cattle in Uruguay: clinico-pathological, epidemiological and genetic survey, and experimental intoxication with *Senecio oxyphyllus.*

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Senecio* spp. transboundary introduction and expansion affecting cattle in Uruguay: clinico-pathological, epidemiological and genetic survey, and experimental intoxication with *Senecio oxyphyllus.

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Abstract

The genus *Senecio* is distributed worldwide, being responsible of poisoning in livestock and humans. Many species of *Senecio* have high invasion and expansion capacity, highly competitive with agricultural and native plant species, causing ecological damage. Particularly in Uruguay, poisoning by *Senecio* have grown exponentially to reach epidemic proportions. Herein we describe Seneciosis as a re-emerging and expanding epidemic disease affecting cattle, by describing clinico-pathological, epidemiological and genetic variation of species involved, as well as an experimental intoxication with *Senecio oxyphyllus*. For this, a study was carried out on 28 cattle farms in Eastern Uruguay, with history of seneciosis from 2010 to 2016. Plants of fifty populations of *Senecio* were sampled, in 2015 and 2016, for identification, analysis of alkaloids and study of genetic variation. In turn, post-mortem examination was performed in cattle of natural and an experimental case to confirm the intoxication, showing microscopic characteristic lesions (hepatomegalocytosis, diffuse fibrosis and ductal reaction). Four species of *Senecio* were identified: *S. oxyphyllus*, *S. madagascariensis*, *S. selloi* and *S. brasiliensis*. In the genetic study, 489 molecular markers of amplified sequence-related polymorphisms (SRAP), associated with species and pasture, were used for genetic variation analysis. There was no statistically significant association between genetic variation determined by molecular markers and population (specimens of same species collected from the same farm), botanically determined species, or geographical origin. The increase of seneciosis in cattle in the last years, the presence of species not identified to the moment with implication in the poisoning outbreaks and expansion of these plants shows that the disease is in an epidemic growing active stage. In turn, the experimental poisoning with *S. oxyphyllus* confirms its chronic hepatotoxic effect, being an emergent species for the region, of high distribution and toxic risk. This latter turned out the main *Senecio* species involved. This case of expansion of harmful plant for animal production and desirable plant species, can be useful as a model of ecopathological characterization, which is likely to occur with other toxic plants in different geographical ranges globally.

Keywords

Plant invasion; hepatotoxicity; ruminants; ecological disturbance; genomic divergence.

Introduction

The genus *Senecio*, belonging to Asteraceae family, with more than 1500 species in the world–(Pelser et al., 2007; Yang et al., 2011), exhibits great environmental diversity (Burrows and Tyrl, 2013). It is established that *Senecio* spp. is one of the most frequent plant-related poisoning in animal production (Stegelmeier 1999; Lucena et al., 2010). Many species are highly competitive with agricultural and native plants, causing production and biodiversity losses (Bradley et al., 2010; Kalusova et al., 2017). Certain *Senecio* species contain pyrrolizidine alkaloids (PA), which act as chemical defenses with great intra- and inter-species variability (Mattocks, 1986, Boppre, 2011). These phytochemical compounds vary in composition and concentration, depending on the genetic constitution of the plant as well as the biotic and abiotic circumstances (Vrieling et al., 1993; Hol, 2011).

The lethal chronic hepatotoxic effect of PAs is well documented, affecting bovines, among other species (Stegelmeier, 2011). There are also sporadic reports of PA poisoning of humans (Prakash et al., 1999). The disease in cattle usually occurs after several weeks or months of continued consumption of *Senecio* plants, primarily in winter when forage is scarce, presenting variable clinical signs, characterized by progressive weight loss and hepatic encephalopathy (Panziera et al., 2017). Anatomopathological findings include diffuse liver fibrosis, hepatocellular degeneration, necrosis and megalocytosis, and ductal proliferation (Cullen and Stalker, 2016).

Some *Senecio* species, such as *S. madagascariensis*, *S. jacobaea* and *S. inaequidens*, have a high capacity of invasion and expansion out of their geographic range, as consequence of human activity, climate change and characteristics of the plants, being an important ecological problem (Wardle et al. 1995; Scherber et al. 2003; Bradley et al., 2010; Celesti-Grapow et al., 2016; Tewes et al., 2018). Plant invasion in new environments by incidental or accidental introduction has generated significant ecological damage and animal health problems (Boppre, 2011). High genetic variation is necessary for evolution of adaptation of non-native plant species in novel ecological conditions (Dietz & Edwards 2006, Oduor et al. 2016). This is why the success of invaders is facilitated by high genetic variability within invasive population, offering an increase in the potential of a population to adapt to the new environments (Müller-Schärer and Schaffner 2008; Tewes et al., 2018). At the same time, ecological processes such as multiple introductions from geographically diverse sources also contribute to the success of dispersion and establishment of invasive populations relative to native populations (Bossdorf 2005; Mader et al., 2016; Tewes et al., 2018). Once *Senecio* plants are introduced into new habitats, they show high colonization capacity with better performance than other native plant species (Mattocks, 1986; Caño et al., 2008; Carvalho et al., 2013) and present higher levels of toxicity than in their native environment (Macel et al., 2004; Kirk et al., 2010; Castells et al., 2014).

These events of appearance and expansion of *Senecio* species have recently been recorded in Rio Grande do Sul (RS), Brazil, near the border with Uruguay (Stigger et al., 2014) and also in the western region of Uruguay (Preliasco et al., 2017). In Australia, New Zealand and North America *Senecio* is considered plague, associated with livestock poisoning and deterioration of desirable native pastures (Coombs et al., 2004; Gardner et al., 2006; Le Roux et al., 2006).

At regional level, in Uruguay, Argentina and southern Brazil the consumption of *Senecio* spp. is the main cause of poisoning in cattle grazing, responsible for up to 50% of all deaths (Riet-Correa and Medeiros, 2001;

Krabbe et al., 2017). Particularly in the eastern region of Uruguay, poisoning by *Senecio* is one of the main pathologies observed in extensive breeding systems, only overcome by bovine piroplasmoses (Dutra et al., 2015; García et al., 2016). *Senecio* spp. is the main toxic plant in the western region of Uruguay as well (personal communication, Rivero, 2017). In recent years, levels of intoxication have grown exponentially to reach epidemic proportions, with 91 outbreaks of seneciosis in cattle registered between 1988 and 2016, 76% of which occurred between 2010 and 2016 (Dutra et al., 2016; García et al., 2018). The outbreaks are spatially correlated, resulting in clusters of high incidence of seneciosis located in the northeast of Uruguay, close to the border with Brazil, and in contrast to the south and southeast of Uruguay, where there is a low incidence of poisoning of cattle by *Senecio* (Dutra et al., 2015, Dutra et al., 2016). The significant increase in *Senecio* poisoning has been associated with a decrease in the sheep population in livestock systems, and the appearance or expansion of *Senecio* species that previously did not prevail nor did exist in the region (Dutra et al., 2016; García et al., 2018).

Regarding *S. oxyphyllus,* there is no experimental confirmation of its effect on cattle in Uruguay. The only intoxication experimentally reproduced in cattle with *S. oxyphyllus* was carried out by Driemeier and Barros (1992) in Brazil. To our knowledge there is no confirmed field intoxication in cattle, since the field outbreaks reported till now involve many *Senecio* species, unable to confirm *S. oxyphyllus* implication (Karam et al., 2011, Krabbe 2017).

There are several studies about intercontinental invasion and expansion of *Senecio* plants; however, few studies, mainly in Europe, have examined the intracontinental expansion (Tewes et al., 2018). To the authors knowledge, in South America there is only one study from genetic perspective that evaluated the intra-continental invasion of *S. madagascariensis* (Mader et al., 2016). Therefore, the present research examines genetic variation, and invasive, epidemiological and clinical-pathological aspects of the expansion of *Senecio* spp. associated with intoxication in cattle grazing in East Uruguay. In turn, an experimental intoxication with *S. oxyphyllus* in cattle is described.

Materials and Methods

1. Seneciosis outbreaks in cattle

1.1. Collection and conditioning of Senecio spp samples

Twenty-eight livestock farms were visited in the eastern region of Uruguay (32º 39’14"S 53º10’58" W), South America, with previous diagnosis of cattle poisoning by *Senecio* between 2010 and 2016, registered by the Direction of Veterinary Laboratories (DILAVE) East Regional. We selected farms that had at least one animal with characteristic microscopic lesions of PA poisoning and botanical identification of *Senecio* plants as diagnostic criteria of seneciosis (Cullen and Stalket, 2016). The visits were made from October to December during 2015 and 2016. For collection of plants, the technique described by Verschave et al. (2015) was used. In each farm, by walking two transrects in the form of "W" within the areas of *Senecio* plants, every 10 steps 1 plant of each species identified as *Senecio* were collected from around the operator, until reaching 3 plants per species identified. Each plant species from the respective farm corresponds to a population, and each 3 plants from each species correspond to individuals. Three green leaves from each 3 plants taken from each *Senecio* species were stored in sealed bags, containing silica gel for drying. Subsequently, plant material was lyophilized in individual Eppendorf tubes (2mL) until DNA isolation. Pyrrolizidine alkaloid content was previously determined (García et al., 2018). The spatial distribution of the species of *Senecio* was classified by observation as different patterns: random, uniform, and aggregated or contagious, following the criteria of Larson et al. (2001) (Figure 1).

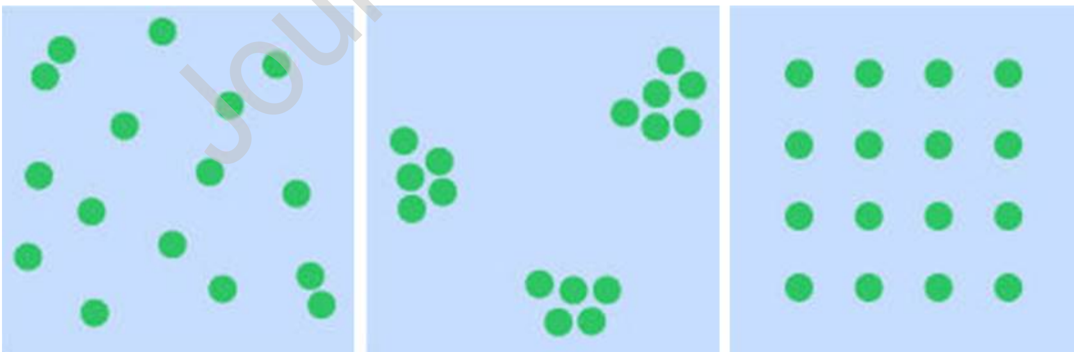

Figure 1. Schemes of spatial distribution of different *Senecio* species classified as random (left), aggregated (middle) and uniform (right). Adapted from Larson et al. (2001).

1.2. Post-mortem examination of cattle

Post-mortem examination was performed in 4 animals (2 heifers and 2 steers), which corresponded to 4 different farms that were included in the

Senecio plant sampling above. Organ samples were collected and fixed in 4% buffered formalin and routinely processed for histological study by cutting sections (5-7 μm thick) then stained with hematoxylin and eosin (HE). The farms presented varied morbidity from 4% to 12%.

1.3. Genetic variation studies

1.3.1. DNA isolation

For DNA isolation, DNeasyPlant Mini Kit (QIAGEN) commercial kit was used following the manufacturer's instructions. The DNA was isolated from 0.2 g of lyophilized vegetal tissue that was previously ground into a fine power in automatic equipment (Geno / Grinder SPEX SamplePrep 2010) at 1000 rpm for 2 min.

1.3.2. Primer selection for SRAP-PCR (Sequence Related Amplified Polymorphism-Polymerase Chain Reaction*)*

The molecular markers for analyzing the genotypic variability were PCR-based Sequence Related Amplified Polymorphism (SRAP, Li and Quiros, 2001). In order to identify the pairs of primers that yielded the highest number of bands that were both reproducible bands, within species and discriminant among species (i.e. polymorphic bands consistently present in samples of the same species), a total of 12 primer pairs combinations were tested (Table 1). The forward primers were marked at the 5' end with VIC or FAM fluorochromes (Alpha DNA, Montreal, Quebec, Canada). The PCR mix (25 μL total volume) consisted of reaction buffer (10x), DNA (25 ng), mixture of dNTPs (10mM), MgCl2 (25mM), Taq polymerase (1 unit) and the pairs of primers (0.5 mM each). The amplification conditions were: initial denaturation at 94°C for 3 min; 5 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 1 min, followed by 35 cycles of annealing temperature at 50°C; and final extension at 72°C for 5 min. PCR amplifications were performed in a standard thermal cycler (BIOER, Hangzhou, China). Sizes of the SRAP amplification products were determined by capillary electrophoresis (Fragment analysis service at Yale University). Genotypes were scored by analyzing fragment sizes using Peak Scanner version 2.0 (Applied Biosystems). Three leaves per plant of three plants of each *Senecio* species (*S. oxyphyllus, S.*

madagascariensis, S. brasiliensis, and *S. selloi*) were analyzed (3 leaves x 3 plants x 4 species) for SRAP primers selection. These sampling was repeated for all the farms (3 leaves/plant and 3 plants/species). The pairs of primers that presented the highest number of consistent bands between repetitions were F02R10 and F02R12, then they were selected for the population analysis.

Table 1. Sequences of primers used in Sequence Related Amplified Polymorphism analysis in *Senecio* spp. samples.

*Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
F02 TGAGTCCAAACCGGAGC**	*R01* GACTGCGTACGAATTAAT
	R02 GACTGCGTACGAATTTGC
	R03 GACTGCGTACGAATTGAC
	R07 GACTGCGTACGAATTCAA
	R08 GACTGCGTACGAATTCAC
	R10 GACTGCGTACGAATTCAT**
	R12 GACTGCGTACGAATTGTT**
	R13 GACTGCGTACGAATTCTG
F03 TGAGTCCAAACCGGAAT	*R01* GACTGCGTACGAATTAAT
	R02 GACTGCGTACGAATTTGC
	R03 GACTGCGTACGAATTGAC
	R07 GACTGCGTACGAATTCAA

** Fluorochrome marked.*

***Primers pairs selected for further population analysis.*

1.4. Population analysis by SRAP

Primers with the highest consistency within species were used for the analysis of the full collected population, under the same PCR conditions as for the SRAP primers selection. The amplified products generated in the SRAP reaction were scored as "1" for presence of band and "0" for absence of band. The association between SRAP markers and species and sampling sites was studied by logistic regression analysis using the MASS package version 7.3-47 (Venables and Ripley, 2002). Each SRAP marker consisted of individual band or combinations of two or three bands. Markers associated to species and farms with a P value <0.001 were selected for the subsequent analyzes. An analysis of molecular variance (AMOVA) was calculated to estimate the distribution of inter- and intra-species genetic variation using the PEGAS 0.1 package (Wright, 1965; Excoffier et al., 1992). The geographical distances between sampled points were calculated from the Global Positioning System (GPS) coordinates using the Spatial Tools package (Peterson and Ver Hoef, 2014). The correlation between matrices of genetic and geographic distances was calculated using the APE 4.1 package, and its statistical significance was obtained through the Mantel test (1967). Statistical comparisons of genetic diversity parameters between included polymorphism content, and percentage of polymorphic bands performed with FSTAT293 (Goudet 2001).

2. Experimental intoxication with Senecio oxyphyllus

2.1. Plant collection and pirrolizidine alkaloid determination

Eighty kilograms of green matter of *S. oxyphyllus* was collected from one farm which had diagnosis of seneciosis in cattle. Plants were dried at room temperature for 48 h in a ventilated and roofed environment and subsequently, they were dried at 60°C for 24 h in oven. The dry plant material was grounded to powder consistency, using a 1mm diameter sieve. A 15 g aliquot was extracted and placed in a 50 mL Falcon tube and sent to the Research Laboratory of Toxic Plants of ARS-USDA (Utah, United States) where the determination of pyrrolizidine alkaloids was performed as previously described (García et al.,2018).

2.2. Intoxication assay

Two calves, Holstein breed, 4 to 6 months old, of 110 and 130 kg, for treated and control animal respectively. The two calves were considered clinically healthy. The treated animal was orally administered by oral-esophageal tube, in daily doses of 4 g dry matter of *S. oxyphyllus* per kg of body weight for 24 days (total 10,560 g DM). Both animals were daily subjected to clinical examination, where clinical parameters such as body temperature, and heart and respiratory rate were measured. At the same time, the animals were observed periodically by the appearance of clinical signs. Blood extraction was performed by jugular puncture on days 1, 3, 5, 8, 13, 15, 17, 19, 22, 24 to analyze the serum enzymes that determine liver function (gamma glutamyl transferase, alkaline phosphatase, albumin, protein). On day 24, the treated animals were euthanized *in extremis*, in accordance with the recommendations of the Uruguayan Honorary Commission of Animal Experimentation (CHEA) (Protocol: Ceuafvet-455). In the post-mortem examination, the collected samples of organs were fixed by immersion in 4% buffered formalin and routinely processed for histological evaluation. Tissues were sectioned at a 5-7 μm thickness and stained with HE.

Immunohistochemistry was performed for immunostaining of epithelial cells of ductal origin in liver tissue, by peroxidase technique and detection kit LSAB2 ® System HRP (Dako) that includes diaminobenzidine substrate-chromogen (DAB, Dako). Anti-Human Cytokeratin 19 primary antibody, clone BA17 (Dako) was used at a dilution 1:100. For antigen retrieval, tissue sections were incubated in citrate buffer solution at pH 6 and maintained in a steamer for 40 min. The technique described by Jain et al. (2010) was used.

The experimental procedures were performed in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines and approved by the Ethics Monitoring Commission on Animal Experimentation (Facultad de Veterinaria, Universidad de la República, Uruguay).

Results

1- Outbreaks

Senecio species identified and its spatial distribution

A total of 50 *Senecio* populations were collected from 28 livestock farms evaluated from 2 regions (Northeast and East center) in East Uruguay (Table 2). Some farms had more than one *Senecio* species, i.e. more than one population of *Senecio*, being more populations than farms. The species morphologically identified were: *S. oxyphyllus* DC, *S. madagascariensis* Poir, *S. brasiliensis* (Spreng.) Less. and *S. selloi* DC (Table 2) (García et al., 2018). The predominant *Senecio* species was S. *oxyphyllus* on 26 farms (García et al., 2018).

Table 2. Abundance of *Senecio* spp. identified from the population survey of 28 farms from northeast and east center of Uruguay during 2015 and 2016. Adapted from *García* et al. (2018)

Region (county surveyed)	Nº farms	Species Identified	Nº Populations
Northeast (Cerro Largo and Tacuarembó)	18	*S. oxyphyllus*	18
		S. selloi	9
		S. madagascariensis	5
		S. brasiliensis	5
East center (Treinta y Tres)	10	*S. madagascariensis*	6
		S. oxyphyllus	5
		S. selloi	1
		S. brasiliensis	1
Total	28		50

Nº: Number

Patterns of spatial distribution according to *Senecio* species were identified. The *S. oxyphyllus* showed an aggregated and random distribution, where individuals are aggregated in dense clusters of plants in several points of the pasture that coalesced, encompassing large areas of the paddocks. *Senecio madagascariensis* presented a random distribution in which, each individual occupied any point of the paddock, with a non-uniform arrangement of individual plants. *Senecio brasiliensis* exhibited an aggregated distribution where there was proximity of individuals with extensive focal dense clustering. *S. selloi* presented a random arrangement, but very low density where few individual plants scattered in space were observed.

Molecular study and genetic variation studies

The primers used for SRAP analysis generated four hundred and eighty nine markers, consisting of combinations of two and three SRAP bands, associated to species and farm ($P < 0.001$). The AMOVA showed that the selected molecular markers did not allow to significantly group by population, species, or geographical origin ($p > 0.05$). However, the greatest genetic variation was due to variation among populations (48.4%, $P = 0.13$), followed by variation among species (28.7%, $P = 0.52$), and among regions (22.9%, P=

0.42). The Mantel test showed no significant correlation between genetic and geographical distance matrices (P = 0.54). There was a significant difference in the proportion of polymorphic content (PC) and richness of bands between species (P<0.05). The greatest genetic diversity based on PC was observed in *S. madagascariensis*, followed by a similar level of PC for *S. oxyphyllus* and *S. brasiliensis*, and finally *S. selloi* with the lowest PC (Table 3).

Table 3. Mean and median polymorphism content (PC), and percentage of polymorphic bands (PC %) in each *Senecio* species from 489 SRAP markers.

Species	Mean PC with 95% CI	Median PC	PC %
S. madagascariensis	0.94 (0.91-0.99)	0.19	24
S. oxyphyllus	0.38 (0.35-0.42)	0.05	15
S. brasiliensis	0.37 (0.34-0.41)	0.24	11
S. selloi	0.24 (0.21-0.28)	0.14	10

Post-mortem analysis of Seneciosis in bovines

The *Senecio* species found in the 4 farms were: 1) *S. oxyphyllus* and *S. selloi*, 2) *S. oxyphyllus* and *S. madagascariensis*, 3) *S. oxyphyllus* and 4) *S. oxyphyllus, S. brasiliensis* and *S. madagascariensis*. The animals exposed to *Senecio* showed progressive weight loss, intermittent diarrhea, tenesmus, depression or marked aggressiveness, followed by death 5 to 7 days after the development of clinical signs (Figure 2). Macroscopically, all animals presented changes in the liver, these included firm consistency, rounded edges with variable degrees of coloration from whitish to gray, and marked white crisscrossed pattern throughout the parenchyma separating into irregular nodules (acinar hepatic accentuation) (Figure 3A). There were ascites, severe mesentery edema and presence of multifocal edematous nodules in the gallbladder with marked wall edema (Figure 3B). The findings were confirmed by microscopic pathology consisting of hepatocellular megalocytosis, diffuse invasive fibrosis and marked ductular reaction (Figure 3C) compatible with PAs poisoning. In one animal, marked *status spongiosus* was observed at the junction of gray matter and subcortical white matter of cerebral cortex.

Figure 2. Adult cow, showing nervous signs, with marked aggressiveness and bad body condition (A) and depression (B).

2-Experimental intoxication with Senecio oxyphyllus in cattle

The concentration of PA in *S. oxyphyllus* specie was found to be 2.6 mg/g, being retrorsine the main alkaloid identified.

The treated animal received a PA dose of 1144 mg/day. After 8 days of treatment, the calf presented progressive drowsiness and thinning with abdominal distension. On the 24th day, the treated animal was euthanized *in extremis* (recumbent and non-responsive). At post-mortem examination, the animal had ascites, mesentery edema and moderately firm liver with whitish capsular surface. On hepatic cut surface, the parenchyma showed discrete white reticular acinar pattern. Microscopically, megalocytosis, mild periportal invasive fibrosis, and moderate bile duct epithelial cells proliferation was observed. The centrilobular veins had perivenular fibrosis. Immunostaining was observed in the cytoplasm of cuboidal cells arranged in tubules of different caliber in portal space and with random distribution forming nests. This confirmed its epithelial origin and in conjunction with microscopic findings was interpreted as bile duct hyperplasia (Figure 3D).

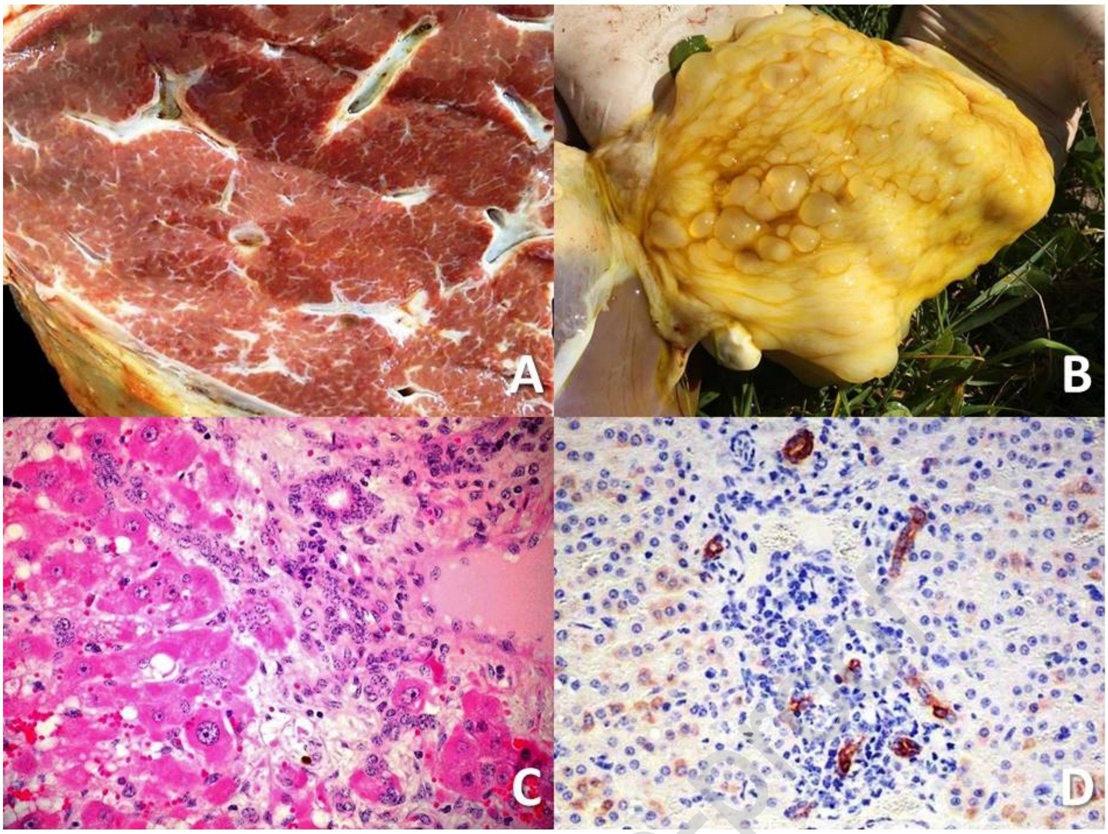

Figure 3. Anatomopathological findings in naturally and experimentally infected cattle with *Senecio* spp. A, Naturally intoxicated adult cow, liver. Firm consistency and marked white crosslink accentuating the hepatic acinar pattern, most like fibrous tissue infiltration. B, Naturally intoxicated adult cow, gallbladder. Multifocal edematous nodules with massive edematous wall. C, Naturally intoxicated adult cow, liver. Presence of hepatomegalocytosis, periportal to invasive fibrosis and ductular reaction with degenerative and necrotic hepatocytes (piecemeal necrosis) (100x, HE stain).D, Experimentally intoxicated calf, liver. Intense immunostaining of bile duct epithelial cells forming multiple nests, confirming ductular reaction. Proliferation that commonly occurs in cattle poisoned with toxic dehydropyrrolizidine containing plants. 20x IHC (anti-cytokeratin 19, dilution 1: 100), diaminobenzidine chromogen and hematoxylin.counter stain reagent.

Discussion

The laboratory data and study of *Senecio* species together with the diagnosis of seneciosis in cattle show that the disease is in an epidemic growing stage in Eastern Uruguay. Explained by the increasing and ongoing outbreaks in the last years, the expansion of *Senecio* species and the re-emergence or appearance of species. Four *Senecio* species identified in the farms with seneciosis in cattle were studied: *S. madagascariensis*, *S. oxyphyllus*, *S. brasiliensis* and *S. selloi* (García et al., 2018). At the same time, the experimental poisoning with *S. oxyphyllus* confirms its hepatotoxic effect, being an emergent species for the region, of high distribution and toxic risk (García et al., 2018). The increase of outbreaks of seneciosis in bovines also occurred in western Uruguay, associated with many outbreaks of *S. grisebachii*, which was recently recognized as toxic (Preliasco et al., 2017), considered highly invasive (Yaber Grass and Leicach 2012).

As in southern Brazil, poisoning by *Senecio* plants in cattle in Uruguay is the main intoxication caused by plants (García et al., 2018; Panziera et al., 2018). The outbreaks showed a spatial correlation in East Uruguay, resulting in clusters of high incidence of seneciosis in cattle located in the northeast of Uruguay, close to the border with Brazil (Dutra et al., 2015). In this problem zone, two highly significant spatial-temporal clusters of high relative risk and long duration were detected. One of them, situated near the border with RS, Brazil, started in 2007, while the other cluster appeared in 2010 in areas farther from the border, to the south of Uruguay (Dutra et al., 2015). Until 2007, seneciosis in eastern Uruguay was presented as sporadic intoxication events, with *S. selloi* being the main responsible, and some foci caused by S. brasiliensis delimited to the mountain ranges of the Cuchilla Grande (Dutra et al. 2010). In the present study, a phenomenon of descending space-time displacement (North-South) of plants at the point of departure of the border with Brazil was evidenced by the clusters (Dutra et al., 2015). These regions are characterized by having soils with low levels of phosphorus and pH (Brazeiro et al., 2012) that favor the emergence and expansion of *Senecio* species (Karam et al., 2011).

Currently, S. o*xyphyllus* and S. *madagascariensis* show an increasing frequency in the epidemic zone that exceeds the historical frequency of S. *selloi* recorded in outbreaks of bovine poisoning in the eastern region of Uruguay (Dutra et al., 2010; García et al., 2018).

Senecio oxyphyllus was classified as an emerging or new species for the eastern region of Uruguay (García et al., 2018). It has been reported as toxic for cattle in the south of Brazil, more than 25 years ago (Driemeier et al., 1992) although it is characterized by low toxicity and presence (Karam 2004). On the contrary, the present study reveals that this species has wide distribution and density in farms associated with cattle mortality. In turn, it contains higher levels of PAs in Uruguay than in Brazil, possibly due to recent colonization (Karam et al., 2004; Kirk et al. 2010; Wolf et al. 2011; Castells et al., 2014; García et al., 2018). The increase of PA levels in invaded areas is reported in *S. inaequidens*, *S. pterophorus* and *S.jacobaea* (Kirk et al. 2010; Doorduin and Vrieling 2011). This is consistent with the “Shift Defense Hypothesis” which argues that introduced plants increase their defense chemicals (Müller-Scharer et al. 2004; Doorduin & Vrieling, 2011).

As for *S. madagascariensis*, it was recognized as a problem in South America since the 90's, with outbreaks of spontaneous intoxication in bovines described in the western region of Uruguay (Preliasco and Rivero, 2011). In recent years it has spread quickly throughout RS, Brazil (Stigger et al., 2014; Panziera et al., 2018). The expansion within Uruguay from west to east region is unlikely because Black river represents a physical barrier that divides the east and west regions from north to south, and may difficult genetic exchange among *Senecio* populations (Haldimann et al., 2003). Therefore, the establishment and spread of *S. madagascariensis* plants in the eastern region might be due to the expansion from RS. In support of this idea, it has been noted the north-south displacement of *Senecio* associated to the occurrence of outbreaks in cattle. However, the recent register of *S. madagascariensis* in RS with increasing incidence could be due to an expansion from northwest Uruguay to Brazil (Villalba and Fernandez, 2005; Stigger et al., 2014). The increasing incidence of seneciosis in cattle in the southern areas of Brazil (Panziera et al., 2018) and Uruguay (García et al., 2018) demonstrates the strong negative impact of the expansion of *Senecio* species, either by transport of seeds contaminated with seeds of *Senecio*, passage of disseminating animals through fecal matter, wind dissemination, and high reproductive or invasive capacity of species associated with a pre-adaptive process (Wiedenfeld, 2011). It is necessary to study the species present in both countries, in order to determine more precisely the migratory route between distant populations.

The high genetic variation presented in *S. madagascariensis* mainly, as well as in *S. oxyphyllus* could be a factor that facilitated the high expansion observed after their introduction into a new range (Le Roux 2006; Müller-Schärer and Schaffner, 2008; Le Roux 2010; Tewes et al. 2018). On the other hand, the low levels of genetic variation of *S. selloi* seem to be a factor that led to its lower expansive capacity (Müller-Schärer and Schaffner, 2008; Hagenblad et al., 2015) and decreased presence in the region (García et al., 2018).

Each species of *Senecio* maintained its own PAs profile (García et al., 2018) suggesting that there is a genetic base (Vrieling et al., 1993; Boppre, 2011; Hol et al., 2011) as well as biotic and abiotic factors (Macel et al., 2004; Kirk et al., 2010) that regulate the pattern of the profile of PAs. Although the genetic component determines the PA profile, it was not possible to differentiate between PA profiles because the molecular markers employed to measure the genetic variation did not allowed discriminating among *Senecio* species. Unlike other authors (Boszormenyi et al., 2009; Nan et al., 2003; Trindade et al., 2009), no relation was found between genetic and chemical diversity within plant population (de Masi et al., 2006; Wolf et al., 2012) indicating that the genetic structure of invasive plant not necessarily give information about its chemical diversity. The difficulty in finding informative molecular markers for *Senecio* has been previously observed (Comes and Abbott, 2001; Pelser et al., 2007), as well as in other invasive plant species (Yu et al., 2014).

The invasive capacity of different *Senecio* species that are generally adapted to ecosystem variations is well known, affecting the risk of invasion plant (Bradley et al., 2010; Celesti-Grapow et al., 2016). This type of invasive plants have various attributes such as short generation times, high fecundity and dispersal capacity, broad environmental tolerance, rapid growth rate and defense to predators that allow their establishment in the face of climate changes that are associated with alterations in the environment (Bradley et al., 2009). The mentioned characteristics are present in some *Senecio* plant species, one of them is the well-known *S. madagascariensis* (Le Roux et al., 2006; Le Roux et al., 2010). Herein *S. oxyphyllus* invasive capacity is showed, though more thorough ecological and botanical characteristics must be studied.

Human-mediated processes favor the dispersion and naturalization of invasive plants, showing a positive relationship between presence of these plants and disturbances of the environment, such as deforestation, grazing and agriculture (Lambrinos, 2004; Kalusova et al., 2017). Particularly, the massive introduction of soybean crops and forestation in Uruguay began in 2010 (Brazeiro et al., 2012), coinciding with the increasing presence of the *Senecio* plant species evaluated in this study. Forestation is a current activity of great importance in the region; almost 1 million hectares of Uruguay are forested, mainly eucalyptus and pine trees. This phenomenon of invasive plants becoming increasingly dominant as disturbance following forest harvests has promoted their presence (Liebhold et al., 2017). This condition leads to the deterioration of desirable native species and gives the opportunity to invasive plants (Bradley et al., 2009), such as *Senecio* species to thrive. Changes in land use or land cover affect ecosystems increase plant invasion, and also directly decrease biological diversity, and therefore limit the potential for diversity loss (Bradley et al., 2010).

Conclusions

The results of this investigation show the expansion of several species of *Senecio* that maybe used as a model of ecopathological characterization for other undesirable toxic plants. In turn, it confirms the toxicity of *S. oxyphyllus*. The high expansion and density of *S. oxyphyllus* together with its relatively high toxicity suggests that it is the main species involved in recent seneciosis in cattle in East Uruguay, with active foci. This shows that *S. oxyphyllus* should be considered a highly invasive plant.

The *Senecio* genus, despite being well known, continues to be a persistent and growing problem worldwide, highlighting an increase in toxicity during its expansion. The high capacity of expansion of species of the genus and the alteration of environments by agricultural activity contributed to the success of naturalization of these invasive species. Genetic variation analysis of toxic plants as a complementary tool is novel for helping to understand species dynamics in invasion or expansion, and thus be able to know and characterize the problem in an integrated way from the animal, the toxic plant and the environment.

The implication in animal health, facing economic damages that destabilize livestock profitability and the reduction of biodiversity with loss of desirable native species makes it necessary to continue the study of these plants. Larger-scale studies are needed with populations in Argentina and Brazil to reach more precise conclusions.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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Dear Prof. Glenn F. King

Editor in Chief, Toxicon

Thank you for your e-mail of our manuscript entitled " *Senecio* spp. transboundary introduction and expansion affecting cattle in Uruguay: clinico-pathological, epidemiological and genetic survey, and experimental intoxication with *Senecio oxyphyllus*" (**TOXCON-D-19-00328**) with its review.

I submit an author statement file outlining all authors' individual contributions:

Juan A. García: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing

Juan E. Rosas: Formal analysis, Resources, Writing - Review & Editing, Supervision

Carmen García y Santos: Resources, Writing - Review & Editing, Supervision

Nicolas Streitenberger: Resources, Writing - Review & Editing

Matías Feijoo: Writing - Review & Editing

Fernando Dutra: Conceptualization, Investigation, Resources, Writing - Review & Editing, Supervision

Looking forward to hearing your decision concerning publication of this paper

September 5, 2019

Highlights:

- *Senecio* spp. plant intoxication is responsible of the most frequent plant-related poisoning in animal production.
- *Senecio* species have a high capacity of invasion and expansion out of their geographic range being an important ecological problem.
- The success of invaders is facilitated by high genetic variability, offering an increase in the potential to adapt to new environments.
- In some countries *Senecio* is considered plague, associated with livestock poisoning and deterioration of desirable native pastures.
- Events of appearance and expansion of *Senecio* species have been recorded.

Sincerely,

Juan Agustín Garcia
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Journal Pre-proof