Environmental impact on biocontrol agents and secondary chemistry of Paterson's curse (*Echium plantagineum*)

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Summary Geographically distinct populations of Paterson's curse (*Echium plantagineum* L., Boraginaceae), found near roadsides across NSW and VIC were surveyed along 3 distinct longitudinal transects in July/August and November/December 2011 for presence of introduced biocontrol agents in shoots, roots or flowers collected from sampled plants. Sampling was conducted twice across 49 geographically distinct sites and associated biocontrol agents were identified and preserved. Survey results showed three of six possible biocontrol agents identified from collected plant specimens, including a crown weevil, a root weevil and a flea beetle. Individual infestation varied across location and clustered distributions of each species were noted across NSW and VIC, possibly due to climatic variation or proximity to former release sites. From each location, a composite sample of shoots and roots was collected. Root periderm extracts were analysed for naphthoquinone content spectrophotometrically and by LC-ESI/MS. Shoot extracts were subjected to solid phase extraction and LC-ESI/MS for determination of pyrrolizidine alkaloids (PAs) and related *N*-oxides (PANOs). Metabolic profiling of 14 possible PAs and PANOs showed their consistent appearance in all shoot extracts with lepthamine *N*-oxide, echimidine-*N* oxide and echumine *N*-oxide predominant. Root extracts contained shikonin, several related naphthoquinones and two PANOs; content varied qualitatively and quantitatively with location and time of sampling.

Keywords Biocontrol agents, crown weevil, root weevil, flea beetle, roots, shoots, naphthoquinones, pyrrolizidine alkaloids, plant defence.

INTRODUCTION

Paterson's curse is an introduced invasive weed species naturalised across much of southern Australia (Piggin 1982). It reproduces by seed, which may persist for seven years or more in the seed bank. It was originally introduced as a potherb in the mid 1800s and later spread as an accidental contaminant of pasture seed and hay. Originally a native of the Mediterranean in Portugal and Spain, Paterson's curse has now invaded over 30 million ha of grazing land in Australia (Piggin 1982, Grigulis *et al.* 2001). In its native range, it occurs in mixtures with other forbs with similar growth habits and morphological traits, rendering it less competitive. However, in Australia it dominates plant communities to the extent that it is estimated to cost the wool and meat industries over A$125 million per year (Carter 2009). Paterson's curse also produces pyrrolizidine alkaloids that cause liver, kidney and lung damage, and eventual death in horses, sheep and cattle (Peterson and Jago 1984). Consumption leads to serious impacts upon wool quality and weight gain (Pratley 1991, Rast 2006).

Although Paterson's curse has been successfully controlled at times by several introduced insect species acting as biological control agents, management of this weed has been sporadic and appears to be dependent upon the successful spread of these agents and optimal environmental conditions (Cowie 2006). Biocontrol agents for Paterson's curse were first imported to Australia by CSIRO in 1979, and again after the *Biocontrol Act 1984*; six agents were released across southern Australia in 1988 and early 1990s. These included a leaf-mining moth (*Dialectica scalariella*), a crown weevil (*Mogulones larvatus*), a root weevil (*Mogulones geographicus*), a flea beetle (*Longitarsus echii*), a stem boring beetle (*Phytoecia coerulescens*) and a pollen beetle (*Meligethes planiusculus*). The most successful biocontrols have been the flea beetle, the crown weevil, and the root weevil. Management of Paterson's curse across Australia has been variable and excessive grazing has led to domination of landscapes by Paterson's curse, especially in droughty soils (Butler and Dowling 2004).

In 2009–2012, we evaluated living Paterson's curse roots with respect to their morphology and production of defence-related compounds. We observed that young roots produced red-coloured naphthoquinones which were localised in outer layers of root periderm. In contrast, mature roots exhibited blackened periderm (Weston *et al*. 2011, 2012). Ethanolic extracts of selected young periderm tissues were red and revealed several unusual naphthoquinones, including shikonin, acetylshikonin, and possibly 1,3 dihydroxy-3-methylanthraquinone, as detected by LC/MS and GC/MS analyses. Mature root extracts were colourless, and contained 1,3 dihydroxy-3-methylanthraquinone

and other related but as yet uncharacterised constituents. Interestingly, both young and aged root extracts exhibited strong inhibition of root growth of annual ryegrass, with young red-coloured root extracts showing greatest phytotoxicity (Weston *et al.* 2012). Strong naphthoquinone inhibition of plant, insect, fungal, and bacterial growth has been shown to be associated with inhibition of electron transport processes, or interference with cell division among other cellular processes (Binder *et al*. 1989, Babula *et al.* 2009, Weston *et al.* 2011). Together with pyrrolizinde alkaloids known to occur in the foliage (Peterson and Jago 1984), it seems likely that naphthoquinones could play an important role in plant defence in regulating herbivory, fungal and microbial relationships in the rhizosphere, as well as allelopathy.

The objectives of this project were to: 1) Survey the spatial distribution of *Echium plantagineum* and three insectan biocontrol agents (*Mogulones larvatus*, *M. geographicus*, and *Longitarsus echii*) across broad areas of New South Wales and Victoria, and compare these with historical records; and 2) identify and quantify the major defensive secondary chemicals of *E. plantagineum* sampled from the geographic range spanned by the biocontrol release sites in New South Wales and Victoria.

MATERIALS AND METHODS

Geographically distinct populations of Paterson's curse were located along or near roadsides across NSW and Victoria (VIC). Populations were surveyed along 3 pre-determined expansive longitudinal transects through NSW and VIC in July/August and November/December 2011. Transects were surveyed to obtain a broad and uniform sampling of Paterson's curse across this region, noted for its Paterson's curse infestation. The first sample transect ranged from Tamworth to Silverton NSW, the second from Lithgow NSW to Mildura NSW, and the third from Cooma NSW to Horsham VIC. At each sampling site, GPS coordinates were recorded, soil and seed samples collected and composite samples of roots, shoots, and insect biocontrol agents collected.

Sampling was performed at each identified site in July/August and November/December 2011. GPS coordinates were noted at each sample site, and plant shoots and roots were collected and evaluated for presence of potential biocontrol agents of importance. Sampling at 45 sites was conducted in survey 1, and additional sites were added for survey 2 for a total of 49. Biocontrol agents were sampled, visually identified and saved for future genotypic evaluation. In addition, five or more plant shoots and roots were collected at each site, extracted separately and analysed for pyrrolizidine alkaloids and naphthoquinones. All tissues were kept cool at 4^{o}C for several days until extraction. Roots were extracted shortly after sampling by peeling thin strips of root periderm and placement in ethanol (1g/10 ml), followed by filtration (0.22 µm syringe filter). Extracts were analysed using absorbance at two wavelengths (493 and 523 nm) for determination of total naphthoquinones, in comparison to a shikonin standard curve (Ozgen *et al.* 2011). The same extracts were also evaluated using an Agilent 6400 LC/MS QQQ system equipped with PDA detector. Samples were injected onto a C_{18} column (Zorbax XDB 4.6 × 50 mm Agilent) using 0.8 ml min^{-1} flow rate with acetonitrile/acidified water (0.1% formic acid) gradient. PDA detection occurred at 254 and 280 nm; ESI/MS evaluation was performed using full scan in negative ion mode. All shoot samples were extracted in methanol (25 g per 250 ml) followed by rotoevaporation to 45 ml. Determination of pyrrolizidine alkaloids was performed using strong cation exchange SPE (Agilent) for concentration of alkaloids followed by LC/MS evaluation as per Colgate *et al*. (2005). Following solid phase extraction, samples were evaporated and reconstituted in 1.5 ml methanol, and subjected to LC-ESI/MS using full scan in positive ion mode. HPLC conditions were as above using a C_{18} column with gradient ranging from 90/10 to 30/70 acidified water/acetonitrile over 20 minutes. Results were replicated over time for verification, and major constituents analysed by comparison with known parent and product ions (Colgate *et al.* 2005, Babula *et al*. 2009, Huang *et al.* 2010). Data presented includes summary results from insect biocontrol surveys and naphthoquinone and pyrrolizidine alkaloid analyses.

RESULTS AND DISCUSSION

The first sampling took place in July/August 2011 when plants were at the rosette stage, except for Victoria where larval emergence was delayed until early September. The second sampling was performed in November/December 2011, over a 5-week period, when plants were flowering. Paterson's curse was found at each sample location by careful searching along roadsides and nearby fields, with the exception of one site at higher elevation near Cooma NSW. Second visits were made to all sampling sites where Paterson's curse was found during the first visit, and specimens were collected from four additional sites based on information from colleagues about the presence of Paterson's curse in Adaminaby, Mt. Selwyn, and Oberon NSW and Beechworth VIC.

Biocontrol survey results The first survey found the three target biocontrol agents at numerous sites

throughout NSW and VIC, with some sites having the presence of only one species, some presenting two species and some with no notable infestations. The second round of sampling 3 months later revealed the presence of the major biocontrol agents at several new sites, but the overall distribution pattern remained largely unchanged from the first round of sampling. In addition, presence of the leaf-mining moth was indicated by the presence of leaf mines on older leaves.

The root and crown weevils were found primarily in the east and south-east portions of the sampling area (most of the sampling area except for the north-west sector) (squares in Figure 1). The flea beetle was found primarily in the south-central region (circles in Figure 1), and the leaf miner was found mainly in the central and western portions (triangles in Figure 1). These data suggest that effective biocontrol agents are not broadly distributed throughout NSW and VIC but rather clustered in these regions, and relative distribution of each is likely associated with climatic conditions across regions as well as concentrated areas of local release during the 1990s. Since the 1990s, major sites of release of these agents have included Tamworth, Dubbo, the Riverina, Albury in NSW; plus Canberra, ACT and many smaller regional release sites. In VIC, major release sites included Wodonga, Mildura and Horsham and scattered regional sites. Other biocontrol agents such as the pollen beetle were not observed. We are currently attempting to locate voucher specimens of crown weevil released across NSW in order to determine if a particular strain was more successful at establishing, as there are two reported strains of crown weevil that were released in the 1980s and 1990s.

Identification and quantification of major plant defence related compounds in Paterson's curse

Methods for identification and quantification of both naphthoquinones and pyrrolizidine alkaloids were successfully developed to analyse both individual and total content in plant samples collected across southern Australia. The methods of Colgate *et al.* (2005) proved invaluable for detection and metabolic profiling of trace quantities of *Echium* alkaloids, including their less stable *N*-oxides. It has been suggested that green vacuolated plant tissues exhibit a higher preponderance of pyrrolizidine alkaloids stored as their hydrophilic N-oxides; in contrast seeds tend to favour storage in a drier environment as parent tertiary

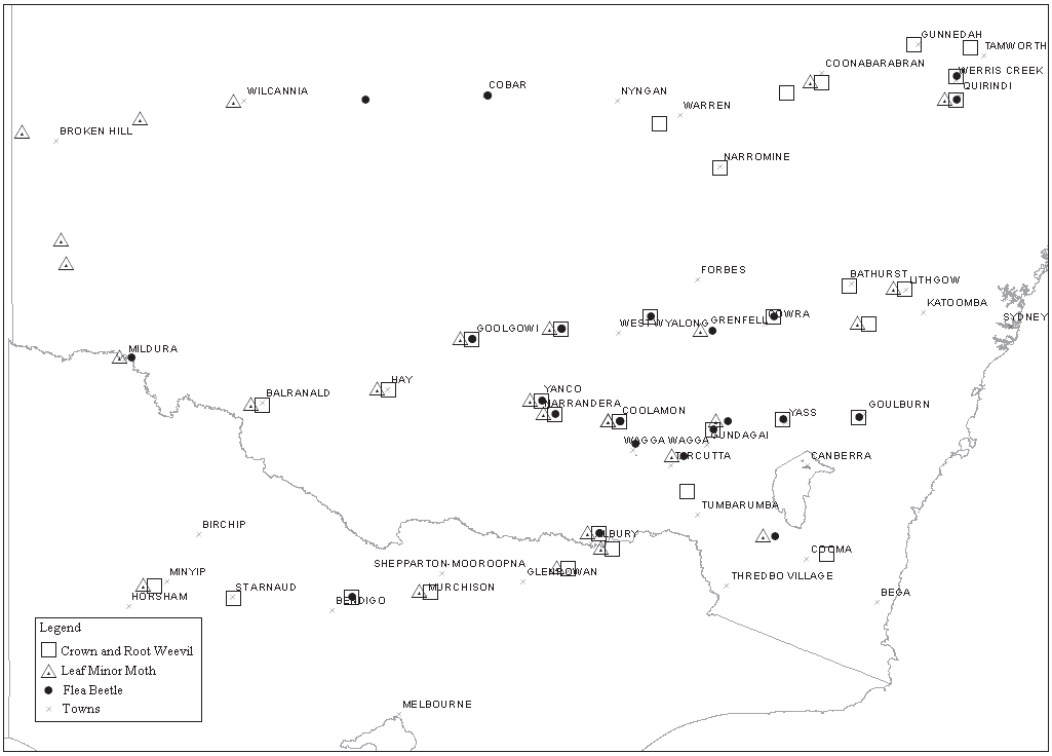

Figure 1. Distribution of the crown weevil and root weevil (squares), flea beetle (circles) and leaf miner (triangles) collected during the survey.

alkaloids (Colgate *et al.* 2005). Much literature exists regarding naphthoquinone and shikonin analysis, and we adapted these methods to determine both total and individual naphthoquinone contents in young red-coloured roots and older roots generating colourless ethanolic extracts.

As described by Colgate *et al.* (2005), the combination of SPE and a strong cation exchanger for alkaloids combined with LC-ESI/MS resulted in a rapid method for profiling plant samples for PAs and their *N*-oxides. The alkaloid profiles of shoot samples collected across Southern Australia were remarkably similar among samples, with up to 14 PA or PANOs readily visualised depending on the sample. This result was similar to that reported, in that 12 of 14 previously observed PAs or PANOs, were found identically in our extracts; however, we observed two that had different parent ions to those previously reported. A typical purified shoot sample containing alkaloids is presented in Figure 2. Major molecular ions were noted for the most prevalent PAs and PANOs; these corresponded to 332 (leptanthine *N* -oxide), 398 (echiumine *N*-oxide) and 414 (echimidine *N*-oxide) which were previously identified by Colgate *et al.* (2005) as major constituents in green tissues. Given the likely presence of diastereoisomers and enantiomers in this mixture, it is likely that not all minor constituents are easily able to be identified. However, 14 major constituents consisting of both PAs and PANOs were observed. Alkaloid levels in dried leaves can range up to 0.25% in *Echium* spp. These compounds have reported activity as liver toxins, carcinogens and genotoxins, besides being implicated in arterial hypertension and neurotoxicity (Colgate *et al.* 2005, Babula *et al.* 2009). Their individual roles on insect biocontrol growth and development have not been directly studied; however it has been reported that pyrrolizidine alkaloids are not considered to impact biocontrol infestation in past findings of CSIRO (A. Sheppard, unpublished communication). Further studies on the direct impacts of PAs and PANOs upon growth and development of major biocontrol agents are planned.

Naphthoquinones that are shikonin derivatives can be identified by their unique red colour, similar to that found in ethanolic root extracts from Paterson's curse periderm peels. Shikonin, molecular weight 288, and its derivatives are frequently used as dyes and colouring agents (Weston *et al.* 2012). In our survey, we observed some root periderms which were not red, and when extracted, these produced colourless or pale pink/peach coloured extracts. Past research showed these extracts were also inhibitory to plant growth, but evidently possessed less shikonin.

This survey revealed that samples collected across locations also varied widely in their red colouration in root periderm tissues, with corresponding differences in naphthoquinone concentrations as determined by LC/MS. Total naphthoquinone content varied up to 100-fold depending on sampling location and time. From comparison with a shikonin standard absorbance curve, red periderm tissue had 8-fold higher concentrations than brown periderm collected from the same plant. Further analysis of roots showed that both red and brown periderm extracts contained varying levels of naphthoquinones, but red extracts contained higher levels of shikonin, deoxyshikonin and acetylshikonin. We also noted trace levels of compounds with molecular ions similar to isoveryl or isobutyl shikonin. As naphthoquinones are known to polymerise routinely when oxidised or heated, it is likely that older roots with blackened periderms also contain naphthoquinone polymers of shikonin

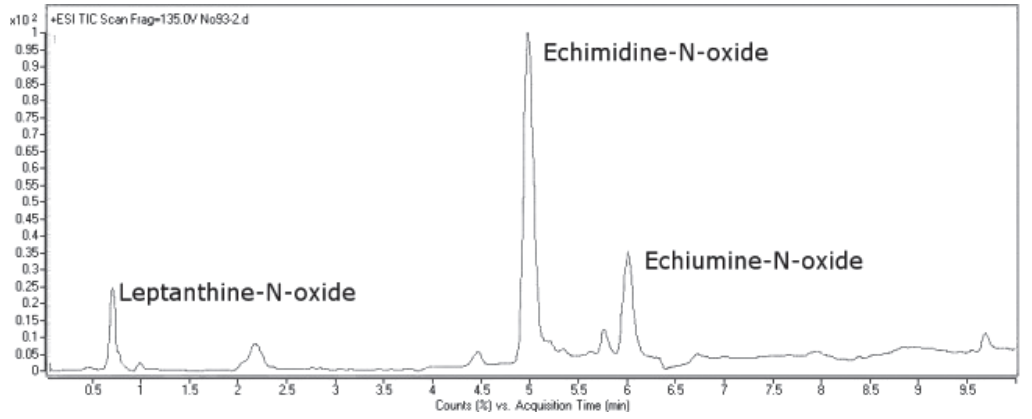

Figure 2. LC-MS trace of the major alkaloids in leaf extracts of Paterson's curse.

derivatives. Interestingly, brown root periderms also contained significant levels of product ions of 458, 487 and 713, indicating possible polycyclisation. Additional MS experimentation will be required to extract and identify these dimers or polymers. We also noted the presence of two predominant PANOs in root extracts, echimidine *N*-oxide and leptanthine *N*-oxide.

Naphthoquinones were first reported in *E. plantagineum* (Weston *et al.* 2011) but related species possessed shikonin, acetylshikonin, deoxyshikonin, isobutyl shikonin, isoverylshikonin and dimethylacryl shikonin, among other derivatives (Huang *et al*. 2010). The ability of *Echium* to produce potent inhibitors that regulate interactions both above and below ground allows us to study the impact of secondary products upon multitrophic interactions, and further investigate their direct impacts upon biocontrol agents introduced for Paterson's curse management.

In summary, populations of Paterson's curse were studied across NSW and VIC and surveyed for the presence of three insectan biocontrol agents and defensive compounds. The insect biocontrol agents were found in clusters throughout NSW and VIC, and defence compounds varied with time of sampling and location, particularly with respect to naphthoquinone content and resultant colouration of root tissues and extracts. Brown periderm extracts from older roots contained less shikonin and derivatives but showed evidence of polymerized naphthoquinone derivatives. Pyrrolizidine alkaloids were observed in all root and shoot extracts and metabolic profiles in shoots were highly consistent among locations. Pyrrolizidine alkaloid profiling offers potential for taxonomic identification or chemo-profiling among related species. Additional studies to assess the relative roles of pyrrolizidine alkaloids and naphthoquinones in plant defence, herbivory and invasion of Paterson's curse across NSW and VIC remain to be conducted.

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