PRODUCTION OF BIOACTIVE NAPHTHOQUINONES BY ROOTS OF PATERSON’S CURSE (Echium plantagineum) – IMPLICATIONS FOR INVASION SUCCESS?

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ABSTRACT
Paterson’s curse roots were studied with respect to their ability to produce coloured secondary products, napthoquinones, in living roots. Young roots produced large quantities of red coloured anthro- or napthoquinones in outer layers of root periderm. In contrast, mature or aged roots exhibited blackened periderm containing accumulations of dark coloured secondary products, possibly due to oxidation or polymerization of these compounds over time. Ethanolic extracts of young root periderm tissues were bright red or pink in colour and contained several unusual napthoquinones, including acetylshikonin, and 1,3 dihydroxy -3- methylanthraquinone, as detected by LC/MS and GC/MS analyses. Mature or aged root extracts were colourless in appearance, and contained 1,3 dihydroxy-3-methylanthraquinone and other likely related constituents. Production of napthoquinones and colour of root extracts was clearly influenced by location of harvest of Paterson’s curse, time of harvest and age of root tissue. Both young and aged root extracts exhibited strong inhibition of root growth of annual ryegrass, with young root extracts showing greatest phytotoxicity. Shikonin at 1 mg/ml was also phytotoxic to annual ryegrass growth, with increasing phytotoxicity noted with increasing shikonin concentration. The role of napthoquinones in plant invasion and their interactions in the plant rhizosphere require further elucidation, as novel napthoquinones exhibit potent antimicrobial, fungitoxic, and phytotoxic activity due to their impact on electron transport and cellular respiration processes.

Keywords: Allelochemicals, antifungal, antimicrobial, bioactive secondary products, periderm, rhizosphere,

INTRODUCTION
Although our understanding of the physiology and metabolism of roots has improved in the last decade, the processes mediated by roots in the rhizosphere, including root exudation and deposition, are not yet well understood (Walker et al., 2003). In addition to the provision of mechanical support and transport of solutes, roots also

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synthesize and secrete a multitude of metabolites over time (Bertin et al., 2003, Brigham et al., 1999). Roots can release these metabolites over time, and they may play roles in defense and rhizosphere signaling (Uren, 2000; Watt and Weston, 2009). Secondary products from root exudates and leachates can also influence soil microbial dynamics in the rhizosphere (Mathesius and Watt, 2010). They also repel herbivores and pathogens, stimulate symbiotic relationships, alter soil properties, and inhibit the growth of competing plants (Bertin et al., 2003; Nardi et al., 2000; Walker et al., 2003).

In terms of ecological interactions, the roots of one plant can compete in the rhizosphere with their neighbours for space, water, nutrients, gases and organic materials that serve as metabolic substrates (McCully, 2005; Ryan and Delhaize, 2001). When roots are under stress, they can react by releasing small molecular weight compounds that are involved in plant defense. These negative forms of communication have received relatively little attention in the literature (Weston and Duke, 2003; Watt and Weston, 2009).

It has been reported that invasive plants use allelochemicals as novel chemical weapons to increase the plant’s ability to interfere with its neighbours. This hypothesis was described initially by Callaway and Aschehoug (2000) as one mechanism influencing exotic plant invasion. Although these interactions are difficult to quantify and their impacts in the field are not well established, many invasive plants appear to utilise allelopathy or root exudation as a means to further compete and increase invasive interference (Douglass et al., 2011).

Paterson’s curse (*Echium plantagineum*) is an introduced invasive weed species naturalized across Australia (Piggin 1982). It was originally introduced as a companion plant in the mid 1800’s and later spread as an accidental contaminant of pasture seed and hay. Originally a native of Portugal or Spain, Paterson’s curse has now been successfully naturalized over 30 million hectares of grazing land in Australia (Grigulis et al., 2001; Piggin, 1982). In its native range, it occurs in mixtures with other forbs with similar growth habits and morphological traits, rendering it less competitive. However, it is now estimated to cost the Australian wool and meat industries over A$125 million per year (Carter, 2009), due to reductions in pasture quality as well as direct toxicity to livestock. Paterson’s curse produces pyrollizidine alkaloids that cause liver, kidney and lung damage, and eventual death in horses, sheep and cattle (Peterson and Jago, 1984). Consumption may have serious impacts upon wool quality and weight in subsequent years (Pratley, 1991; Rast, 2006). Management of this weed has been sporadic and appears to be dependent upon successful spread of biocontrol agents and optimal environmental conditions (Cowie, 2006; Scott and Kenneally, 1981).
To date, little attention has been focused on the root system of Paterson’s curse and below ground interactions. The plant exists either as an annual or biennial (Grigulis et al., 2001), and can survive long periods of drought due to a deep taproot. We have also observed, as widely reported, that the plant germinates throughout the winter months in NSW, forming flat rosettes which persist for months or longer, supported by a deep tap root and smaller lateral roots. Upon collection, we noted the strikingly dark colouration of the taproot and sometimes its lateral roots. Thus, we performed a series of microscopic and chemical evaluations and herein, report on our observations.

MATERIALS AND METHODS

Microscopy

Paterson’s curse plants were collected from field sites in Coolamon and Wagga Wagga NSW from 2008-2011. At each location, 10-20 plants were collected in June and July (mid-winter) and again in August and September (early spring), once flowering had commenced. Plants in each location grew in mixed pasture settings of annual and perennial grasses, along with common weeds. Both sites were characterised by alluvial soils with primarily Devonian granite composition, classified as red earth soils with a loamy sand base and low percentage of clay. After removal, plant roots were washed thoroughly in tap water and blotted dry before processing. Plants were observed microscopically in 2008-2011 using a standard dissecting microscope (Zeiss Corporation, Jena FRG) or an optical bright field light microscope with fluorescent imaging (Nikon A1 confocal microscope, Nikon Australia, Lidcombe NSW 2141). Roots were dissected using a scalpel blade and trimmed to form thin cross sections of tap roots as well as lateral roots. Longitudinal sections of tap and lateral roots were also evaluated.

Chemical extraction

In 2008-2011, roots of Paterson’s curse plants were collected before and after flowering as described above. After washing, thin peridermal peels of both tap and lateral roots were collected by removing periderm using a sharp scalpel blade. Root periderm could be distinguished from underlying tissues due to their colour which was blackened or bright red/violet in most cases. Older tap roots were darkened in appearance and were separated from younger tap and lateral root tissues which were red/violet in colour. At each sampling, 2 g fresh weight of older tap roots, younger tap roots and lateral roots were collected and processed separately from each location. After weighing, root samples were extracted in 100% ethanol (2 g tissue/5 ml ethanol). Young roots were extracted a second time to further
remove coloured constituents. All extracts were filtered through 0.2 µm syringe filters.

**Chromatography**

In 2008-2010, extracts were subjected to thin layer chromatography (TLC) using silica gel preparative plates treated with fluorescent detector developed in 6:1 chloroform:methanol. In 2010-2011, extracts were further subjected to evaluation using gas chromatography coupled to mass spectrometer equipped with a 30 m Agilent DB 5 column (GC/MS; Agilent 6890N benchtop, standard conditions) and also liquid chromatography/mass spectrometry (LC/MS) (Agilent Triple Quad MS system / UPLC). Mobile phase for all LC/MS analyses was a gradient of 50% acetonitrile:water to 100% acetonitrile over 15 minutes at 1ml/minute on an mRP 18 column (Agilent, 0.5 x 100 mm) under positive and negative ionization conditions. Standards were obtained for comparison/validation and included shikonin (0.1mg/ml) and acetylshikonin (0.1 mg/ml) (Sigma Aldrich Pty, Sydney Australia).

**Chemoassay**

Small glass petri dishes were used to assess the inhibitory activity of young, mature and old root extracts, as well as shikonin, upon radical elongation of annual ryegrass seedlings. Annual ryegrass seed was collected locally in 2010. Young, mature and aged, darkened root extracts were obtained by collecting Paterson’s curse in May 2011 in Coolamon NSW and preparing 2 g of fresh washed roots as described above. Roots were placed in 5 ml of ethanol and each extract was filtered using a 2 µm filter to remove debris and particulates. Filtered extracts at 0.5 ml quantities were placed on filter paper (Whatman 10) placed in glass petri dishes. Ethanol was allowed to evaporate for 1 hour. Ten ryegrass seeds were placed in each dish, followed by the addition of 0.5 ml of distilled deionized water. Treatments were replicated three times for a total of 30 seedlings measured per treatment. Dishes were stored at 24°C temperature for 5 days and radical elongation was measured and recorded. Seedling growth inhibition was assessed in comparison to the untreated control, as a percentage of radical length inhibition.

**RESULTS**

In 2008 and 2009, Riverina NSW experienced significant drought. Collected plants in 2008-2009 were generally smaller with roots darkened in appearance, yielding highly coloured extracts. In 2010 and 2011, moisture availability in soils was improved due to rainfall, and plant growth was more luxuriant, but roots and extracts less coloured. In 2008 and 2009, young root extracts were pink rather than red, indicating limited extraction of coloured constituents (Table-
Mature root extracts were uncoloured in each year and season of collection. As plants matured, roots produced red extracts after flowering (August/September) in comparison to root extracts collected in June/July.

Upon closer inspection of roots under a dissecting microscope, the peridermal layers of root tissues were coloured, with underlying tissues white or cream coloured. The peridermal layer was black or brown in oldest roots, with younger taproots and laterals exhibiting red coloured periderm, due to the presence of secondary products. In 2008-2009, this layer was bright red in younger root tissues. Upon closer examination, the periderm consisted of several layers of highly coloured cells, with each cell exhibiting reddened vacuoles or vesicles with red cellular constituents. In the oldest portion of the root, periderm layers had begun to disintegrate, exhibiting cellular extrusion and sloughing. These cells contained black constituents. Extracts of young roots were pink or bright red, depending on concentration of coloured constituents. Extracts from the oldest roots remained colourless, despite the darkened appearance of root peels; dark constituents were insoluble in ethanol. Young root extracts contained multiple fluorescent constituents and a high concentration of two to three napthoquinones, depending on the sample. Analysis by GC/MS showed 20 major and minor constituents, including napthoquinones.

LC/MS also confirmed presence of napthoquinones. Under negative ionization mode, several compounds with molecular masses of 204 to 383 were detected. Paterson’s curse, a member of the Boraginaceae, produces napthoquinones similar to those of the related Lithospermum spp. (Brigham et al., 1999). Root extracts of Paterson’s curse contained compounds which co-eluted with shikonin standards, suggesting their presence in the extracts. Additional GC/MS study revealed 1,8 dihydroxy-3-methylanthraquinone, a napthoquinone with a molecular weight of 383. Extracts of younger roots also contained anthraquinones. LC/MS analysis of coloured extracts revealed the presence of small amounts of shikonin (molecular weight 288), and larger amounts of acetylshikonin (330) and a related compound (356), along with unknowns. Aged roots did not contain shikonin but other anthraquinones were present. GC/MS analysis of these samples revealed the presence of 1,8 dihydroxy-3-methylanthraquinone.

Young root extracts proved highly inhibitory to seedling root growth of annual ryegrass in petri dish assays (70% inhibition), and mature and aged root extracts were also inhibitory. Shikonin also exhibited phytotoxicity at the concentration of 1mg/ml (50% inhibition), and 0.1 and 0.01 mg/ml treatments still produced observable inhibition. A single wash with ethanol removed the majority
of inhibitors, as a second subsequent extraction resulted in limited inhibitory activity (17%).

Table-1. Level of total shikonin derivatives in extracts of young Paterson’s curse roots in ethanol, as influenced by season and year, by colourimetric evaluation of 2 g fresh tissue/ 5 ml ethanol. Dark red colour indicates high concentration of total shikonins whereas colourless solutions indicate low levels of shikonins. NA=not available.

<table>
<thead>
<tr>
<th>Year</th>
<th>June/July collection</th>
<th>August/September collection</th>
</tr>
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<tbody>
<tr>
<td>2008</td>
<td>Pink</td>
<td>dark red</td>
</tr>
<tr>
<td>2009</td>
<td>deep pink</td>
<td>dark red</td>
</tr>
<tr>
<td>2010</td>
<td>Colourless</td>
<td>deep pink</td>
</tr>
<tr>
<td>2011</td>
<td>Pink</td>
<td>NA</td>
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</tbody>
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DISCUSSION

Based on review of the published literature, the production of red coloured secondary products in plants or animals is rare with only a few uniquely bright red in colour, similar to those in Paterson’s curse roots. Besides anthocyanins, haeme or haemoglobin-like molecules, there exist anthraquinones or napthoquinones which are red and, upon oxidation, purple in colour. These napthoquinones are produced by roots of several Boraginaceae. The production of red-coloured constituents, shikonin derivatives or napthoquinones is reported in cultures or living roots of *Lithospermum erythrorhizon*, a member of the Boraginaceae (Tabata and Fujita, 1985). Shikonin is a red precursor to many related active napthoquinones, and its production can be induced in root cultures, by stressful conditions or pathogenic fungi (Brigham *et al.*, 1999; Tabata and Fujita, 1985).

Napthoquinones are used as dyes, colourants or as medicinal products. Some related naptho- or furanoquinones have been reported to be cytotoxic, anti-inflammatory and antimicrobial (Brigham *et al.*, 1999). Napthoquinones such as juglone function as allelochemicals and inhibit plant growth, and have also been shown to inhibit electron transport or respiration (Binder *et al.*, 1989). Shikonin and its derivatives were shown to be strongly elicited in *Lithospermum* root cultures by the presence of fungi such as *Rhizoctonia solani*, *Pythium aphanidermatum* and *Nectria hematococca* (Brigham *et al.*, 1999). Root cultures contained several napthoquinones with strong antimicrobial activity against cultured soil microbes. *In situ* these compounds are produced and released by living root hairs of *L. erythrorhizon*, which turn red as compounds accumulate (Brigham *et al.*, 1999; Tabata and Fujita, 1985).
Figure 1. Clockwise from top left. Paterson’s curse taproot showing characteristic darkened or reddish appearance of root periderm in 2009. Root periderm cross section from young Paterson’s curse root, collected in 2010 and viewed with confocal microscopy, 40X. Root periderm cross section showing close up of periderm cell and red coloured constituents with confocal microscopy, 100X. Light microscopy of Paterson’s curse root periderm cross section, 80X.
We observed that red napthoquinones are produced by living Paterson’s curse roots, and localised in the periderm of young roots. Upon aging, the periderm becomes blackened and coloured constituents are insoluble in ethanol. The root extracts of young roots are also highly inhibitory to seedling growth, in assays with annual ryegrass, a common competitor in NSW pastures. Young root extracts were inhibitory at 100 µg/ml of napthoquinones or less, judging by colour and concentration of these in extracts, which were later subjected to LC/MS and GC/MS. Napthoquinones present included acetyl shikonin, shikonin and 1,8 dihydroxy-3-methylanthraquinone. Environment and genotype also influenced production of these constituents. Extracts of aged roots were also inhibitory and contained napthoquinones but were uncoloured. Additional studies will further elucidate structures of active constituents in extracts of both young and old roots.

Both GC and LC/MS studies indicated the presence of shikonin derivatives, similar to those produced by *Lithospermum* spp. (Brigham et al. 1999; Tabata and Yashita 1985). In *Lithospermum* spp., napthoquinones and shikonin derivatives were produced in association with the endoplasmic reticulum and later transported to the cell membrane where they bind to cell wall constituents, turning the entire cell a reddish colour (Tabata and Fujita 1985). We noted the same pattern of distribution in younger roots of Paterson’s curse. In contrast to *Lithospermum* spp. however, these compounds are evidently produced in the periderm of Paterson’s curse but not in living root
hairs as is the case in *Lithospermum* spp. (Brigham *et al.* 1999). Root extracts contain not only shikonin, but several related compounds, including acetylshikonin and another naphthoquinone, identified based on comparative analysis with published reports elucidating generation of molecular ions and fragmentation patterns using LC-MS (Brigham *et al.* 1999; Tabata and Fujita 1985). Shikonin production is strongly affected by stress, including stress induced by light, temperature and pathogen exposure plus chemical stressors, such as proteins (Brigham *et al.* 1999; Dixon and Pava 1995). We observed site and seasonal variation in production of naphthoquinones. Plants collected in midwinter versus spring produced significant levels of extractable naphthoquinones, with increased concentration noted over time.

Naphthoquinones are novel secondary products with potent antimicrobial, cytotoxic and antifungal properties. This project has demonstrated existence of naphthoquinones in Paterson’s curse roots and root extracts. Extracts exhibited strong phytotoxicity to annual ryegrass seedlings. Their specific role in contributing to invasive success of Paterson’s curse in Australia remains to be further elucidated.

REFERENCES CITED


