1   *Short communication*

2   SUBTLE, SEASONAL AND POTENTIALLY IMPORTANT: ARE PHYTOTOXIC FOLIAGE LEACHATES

3   OVERLOOKED IN PLANT INVASION STUDIES?

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21 **Abstract** – Invasive species pose a threat to global biodiversity. Recently there has been a growth in research

22 into the chemical aspect of invasions, with some plants reported to introduce compounds to the soil which

23 impair the growth of competing native plants. One such species is *Rhododendron ponticum,* which has become

24 a damaging invasive shrub in Britain. Here we investigate whether water-soluble compounds introduced from

25 *R. ponticum* leaves in Ceredigion, Wales, suppressed seed germination, and whether this varied temporally.

26 Leachates from leaf material collected monthly (March to July) were analysed for their chemical composition

27 by liquid chromatography-mass spectrometry and *Lactuca sativa* bioassays. In total, 19 compounds were

28 identified, displaying both qualitative and quantitative seasonal variation. Total concentrations peaked in June,

29 however *L. sativa* germination inhibition was only found in leachates from April and May (15% and 17%

30 respectively). The study provides evidence that phytotoxic chemical introduction from *R. ponticum* leaves is

31 seasonal and most bioactive at a time of year when the seeds of other species germinate. We propose this is

32 one of several mechanisms including shading, lowering of soil pH and the deposition of thick litter, that this

33 species exploits when in competition with other species. We argue that for studies on the phytotoxicity of

34 invasive plants, there is a need to consider temporal variation in the rate and quality of bioactive chemicals

35 produced and released, local precipitation rates, persistence of chemicals in the soil plus synergistic

36 interactions between individual chemicals.

37 **Key Words** – Phenolics, invasive, secondary metabolites, seasonal, inhibitory.

38   INTRODUCTION

39 Invasive species are increasingly damaging habitats worldwide, driven by the increased movement of goods

40 and people due to globalisation (Perrings et al. 2005). The ecological factors behind plant invasions are well

41 studied. Recent years however have seen a surge in interest in the chemistry of plant invasions, partly

42 prompted by the "novel weapons hypothesis" proposed by Callaway and Aschehoug (2000). Many species are

43 reported to possess such chemical traits, for example *Rhododendron ponticum* L., which has become a

44 problematic invasive shrub in the UK following its introduction from Spain during the 18th century (Cross 1975).

45 In addition to its suitability to the British climate, some studies suggest *R. ponticum* also alters soil conditions

46 by introducing bioactive secondary metabolites to the soil which impair the growth of competing native plants.

47 Rotherham and Read (1988) found *Festuca ovina* L. (sheep's fescue) root elongation was inhibited when

48   growing in *R. ponticum* soil, an effect which was not alleviated by the addition of nutrients or water. This

49   suggestion of phytotoxic compounds is supported by laboratory bioassays on litter leachates from another

50   *Rhododendron* sp. (Nilsen et al. 1999).

51   Our investigation aimed to quantitatively and qualitatively study the seasonality of phenolic compounds in *R.*

52   *ponticum* foliar leachates by liquid chromatography-mass spectrometry (LC-MS^n), and the effect of these

53   compounds on *Lactuca sativa* L. germination. It was hypothesised that these compounds are delivered via

54   leachates from *R. ponticum* canopies and fallen leaf litter, with concentrations highest in spring (March, April

55   and May) when the majority of seeds from other species germinate. Leading on from this, it was also

56   hypothesised that leaf leachates would be most inhibitory to *L. sativa* during these spring months, due to the

57   reported phytotoxicity of some phenolic compounds.

58   METHODS AND MATERIALS

59   *Site description* Sampling was conducted at a mixed woodland called Pwll Crwn (OSGB36 grid reference:

60   SN623283), Ceredigion, Wales. This site was cleared of *R. ponticum* between May and July 2013 but plants had

61   regenerated by March 2016. Samples were taken from 0.5 m stems regenerating from previously cut stumps.

62   *Leaf collection, phenolic compound analysis and bioactivity* Leaf leachates were collected monthly (March to

63   July) from five independent randomly selected plants. Leaf extracts were used as opposed to soil extracts as it

64   allowed the study to focus purely on the effect of the compounds derived from *R. ponticum*; soil extracts

65   would contain compounds from other plants and microbes, as well as vary seasonally in terms of chemical

66   properties. Separate plants were sampled each month to avoid any effects of leaf removal on subsequent

67   secondary metabolite content. Extracts containing 3 g of sample tissue placed in 15 mL distilled water (1:5)

68   were prepared using the method described in Nilsen et al. (1999). After storing at 4°C for 24 hours, 500 mg

69   Sep-Pak C_{18} 3 cc Vac RC cartridges (Waters Ltd, Wexford, Ireland) were used to partially purify the extracts

70   prior to LC-MS^n analysis. Following this, the extracts were evaporated to dryness with a vacuum centrifuge

71   (Jouan RC1022, Nantes, France), before resuspending in 200 µL 70% methanol.

72   The processed extracts were analysed by LC-MS^n on a Thermo-Finnigan LC-MS system (Thermo Electron Corp.,

73   Waltham, USA), consisting of a Finnigan Surveyor PDA Plus Detector, a Finnigan LTQ linear ion trap with ESI

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74   source and a 4 µm C_{18} Waters Nova-Pak column (3.9 mm × 100 mm). A constant temperature of 5°C was

75   maintained for the autosampler tray, and 30°C for the column. The injection volume was 10 µL. The column

76   was equilibrated with 95% of solvent A (water/formic acid; 100:0.1, v/v) and 5% solvent B (methanol/formic

77   acid; 100:0.1, v/v). The percentage of solvent B increased linearly to 60% over 65 minutes, at a flow rate of 1

78   mL/min.

79   MS^2 fragmentation data were used to identify compounds in negative ion mode. Compound concentrations

80   were calculated using standard solutions of known volume for each of the chemical groups identified where

81   available or the aglycone where compounds were not commercially available. Unknown compounds were

82   quantified based on relative abundance.

83   Following on from these chemical analyses, *L. sativa* (var. Buttercrunch) germination bioassays were

84   conducted to investigate the impact these leachates have on plant growth, based on the method described by

85   Nielsen et al. (1999). This species was selected as it is a standardised indicator species commonly used in

86   studies on phytotoxic compounds. 20 seeds were evenly spaced on moistened (with 2 mL extract) filter paper

87   in Petri dishes (55 mm); controls had the same volume of distilled water. Mean germination percentage (plus

88   root and shoot length) per plate was recorded after incubation at 20 °C for seven days.

89   *Statistical analyses* Statistical analyses were conducted in SPSS (23.0, IBM, USA) using multiple regression and

90   one-way ANOVA followed by the LSD post-hoc test (P < 0.05). Mean germination percentage was arcsine

91   transformed prior to statistical analysis, whilst the concentration data were log transformed prior to multiple

92   regression analysis.

93   RESULTS

94   In total, 19 different phenolic compounds were detected, with all but two being identified (Table S2). Most

95   compounds were present across all five months (March to July) with total concentration peaking in June (Fig. 1

96   and Table 2S) before decreasing significantly to July (P < 0.001). Whilst total phenolic concentration was

97   highest in June, inhibition of *L. sativa* germination was highest in April and May (P < 0.001) (Fig. 1). Of the 19

98   compounds detected in the leachates, only quercetin was found to be significantly more concentrated during a

99   month where germination inhibition was observed; its concentration was higher in May compared to March

100   and April (P < 0.05), whilst it was not present during June or July (Table 2S). A multiple regression analysis

101   showed none of the 19 compounds had a significant relationship with germination (Table 1S), with only

102   quercetin showing a near-significant negative relationship (P = 0.061).

103   DISCUSSION

104   Total phenolic compound concentration was significantly higher in June, contrary to our original hypothesis.

105   Black et al. (2011) found seasonal variation in total phenolic compound concentration in the related species *R.*

106   *tomentosum* to be closely correlated to day length in the Canadian arctic (Black et al. 2011). Whilst total

107   phenolic compound concentration in our study coincided with the longest day length in June, the significant

108   decrease in concentration from June to July suggests that day length was not solely responsible for the

109   seasonal variation observed.

110   Other functions these compounds have within plants could also be responsible, including herbivore defence

111   (Bennett and Wallsgrove 1994). Previous studies have shown young leaves and reproductive tissues have

112   higher concentrations of secondary metabolites such as phenolics compared to older leaves, as they lack

113   physical strength and are thus vulnerable to herbivory (Bennett and Wallsgrove 1994). This implicates that

114   phenolic compound concentration should have been highest in March when the leaves were young,

115   decreasing as the leaves gain physical strength as they age. Again however, our results contrast this, with total

116   concentration was highest in June.

117   Despite total phenolics concentration being highest in June, *L. sativa* germination inhibition was highest during

118   the spring months, consistent with our second hypothesis. Only quercetin was found to be significantly higher

119   during one of these months however (May), a compound which was observed by de Martino et al. (2012) to

120   inhibit the germination of garden cress. Other putative phytotoxins were also detected during May, including

121   catechin, gallic acid and dihydromyricetin (Thorpe et al. 2009; Castro et al. 2010; Weidenhamer et al. 2013).

122   However, these were present at higher concentrations in June (P < 0.05), and multiple regression analysis

123   indicated that none were significantly correlated with suppressed germination.

124   These findings suggest that the observed germination suppression was not caused by an individual compound,

125   rather it may be the result of complex interactions between compounds. The lack of a clear relationship

126   between the seasonal variation in phenolic compound concentration and inhibition may also be influenced by

127   the other roles phenolic compounds have within plants, as previously discussed.

128   The lack of understanding surrounding the roles of phytotoxic compounds in plant ecology is partly due to the

129   complexity involved in designing an experiment which isolates the effect of the compounds from other

130   environmental factors. Previous studies have used activated carbon which is reported to adsorb these

131   compounds, however the addition of activated carbon may confound results by altering soil chemical

132   properties (Weißhuhn and Prati 2009). Complexity is further increased by potential interactions with soil

133   microbes, which are reported to mediate the effect of phytotoxic compounds, however this was not observed

134   by Duke et al. (2009). We avoided using soil extracts in the current study, as the focus was purely on the

135   synthesis of these compounds, in terms of both quantitative and qualitative seasonality, and the implications

136   variations in these have on future studies in the field of chemical ecology. Whilst we acknowledge that soil

137   interactions are not accounted for, we argue that the experimental design allowed the isolation of the effect

138   from compounds released by other plants, in addition to seasonal variation in soil chemistry and

139   environmental conditions.

140   We suggest that phytotoxic leaf leachate is just one of several interacting mechanisms (including shading,

141   deposition of a deep litter layer and root exudation of phytotoxic compounds) that cumulatively contribute to

142   the competitiveness of *R. ponticum*. The timing of inhibition appears to coincide with the germination and

143   early growth period for many competing native seedlings.

144   The importance of phytotoxins in plant invasions is questionable, yet any such conclusions should consider

145   seasonal variation, initial bioactivity and persistence in the soil. For example, the role of the phytotoxin

146   catechin released from roots of the North American invasive *Centaurea stoebe* has been a topic of debate

147   (Duke et al. 2009). Conflicting findings of studies on catechin in *C. stoebe* soils highlight the importance of

148   considering temporal variation when investigating phytotoxcity mechanisms in invasive species, as the rate of

149   production and release of individual phytotoxic compounds may be seasonally dynamic. Additionally, the

150   concentration reaching the soil or leached away would depend on the intensity of recent precipitation.

151   Compound persistence in the soil would also be dictated by microbial breakdown and adsorption processes in

152   the soil (Blair et al. 2006; Weidenhamer et al. 2013). Finally, individual compounds may not act in isolation and

153   likely interact synergistically even at low concentrations.

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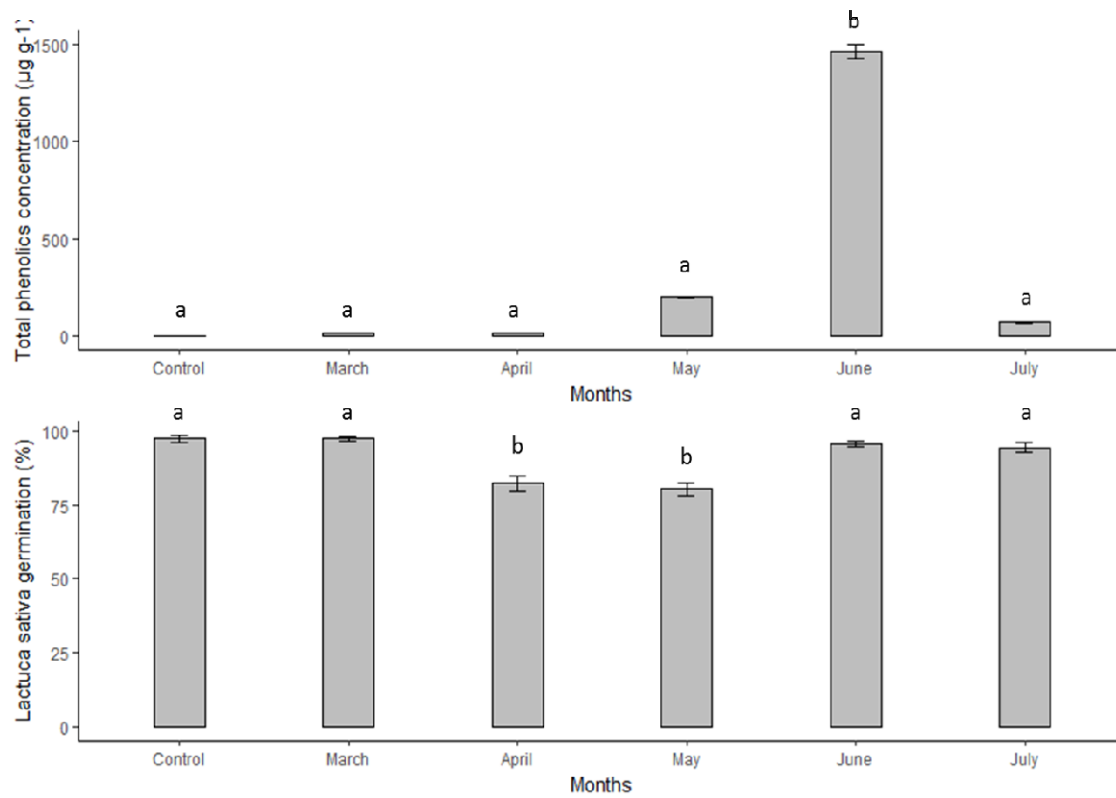182   plants. Basic Appl Ecol 10:500–507 . doi: 10.1016/j.baae.2008.10.009

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FIGURES

Figure 1: The monthly (March to July) total phenolic compound concentration ($\mu g\ g^{-1}$) of the *R. ponticum* leaf leachate, and the mean germination percentage of the *L. sativa* seedlings growing in the leachate. The error bars represent the standard error of the mean. Means with common letters did not differ significantly ($P <$ 0.05) following one-way ANOVA and LSD analyses.

SUPPLEMENTARY DATA

Table 1S: The R^2 value and *P* value for each of the 19 compounds following a multiple regression analysis in the SPSS statistical software (significance level *P* < 0.05). Prior to analysis, the percentage germination data was arcsine transformed, whilst the concentration data for the compounds was log transformed.

Compound	R^2	*P* value
Gallic acid	0.116	0.426
Gallocatechin	0.255	0.339
Methyl gallate	0.371	0.270
Catechin	0.009	0.494
Dihydromyricetin	-0.130	0.418
Digallic acid	0.369	0.270
Epicatechin	0.552	0.167
Coumaric acid	0.118	0.425
Rhododendrin	0.368	0.271
Taxifolin-*O*-pentoside	-0.251	0.342
Epicatechin gallate	0.552	0.167
Dihydromyricetin-*O*-galloyl hexoside	0.373	0.268
Quercetin-*O*-galloyl hexoside	0.368	0.271
Myricetin-*O*-rhamnoside	0.325	0.297
Unknown phenolic compound 1	0.501	0.195
Quercetin	-0.778	0.061
Unknown phenolic compound 2	0.368	0.271
Quercetin-*O*-pentoside	0.164	0.396
Quercetin-*O*-rhamnoside	0.354	0.279

Table 2S: Mean concentrations of the phenolic compounds detected in *R. ponticum* leaf leachate per tissue fresh weight ($\mu g\ g^{-1}$) for each month. Total phenolics are also shown. Means with common letters were not significant different (P < 0.05) following One-way ANOVA; LSD Post Hoc Test.

Compound and retention time (min)	March ($\mu g\ g^{-1}$)	April ($\mu g\ g^{-1}$)	May ($\mu g\ g^{-1}$)	June ($\mu g\ g^{-1}$)	July ($\mu g\ g^{-1}$)
Gallic acid (1.6)	0.012 ± 0.015 a	0.263 ± 0.24 a	39.86 ± 20.111 a	648.005 ± 153.272 b	24.542 ± 9.722 a
Gallocatechin (3.4)	0.01 ± 0.01 a	0.087 ± 0.087 a	0.316 ± 0.205 a	5.106 ± 5.097 a	0
Methyl gallate (5.1)	0.011 ± 0.011 a	0	0	29.116 ± 9.688 b	0
Catechin (7.2)	0.007 ± 0.007 a	0	12.842 ± 12.819 a	131.405 ± 55.755 b	0
Dihydromyricetin (9.3)	0.001 ± 0.001 a	0.029 ± 0.019 a	14.979 ± 9.386 a	37.922 ± 3.507 b	0
Digallic acid (11.6)	0.007 ± 0.007 a	0	0	10.905 ± 5.071 b	0
(Epi)catechin (12.7)	0.062 ± 0.062 a	0	0	0	0
Coumaric acid (13.2)	0	3.376 ± 0.869 a	1.479 ± 0.911 a	10.182 ± 10.164 a	16.777 ± 4.219 a
Rhododendrin (14.9)	0	0	0	46.936 ± 18.49 a	0
Taxifolin-*O*-pentoside (16.9)	1.507 ± 0.704 a	1.081 ± 0.749 a	81.168 ± 61.567 a	41.449 ± 11.662 a	5.180 ± 1.611 a
(Epi)catechin gallate (17.6)	7.510 ± 7.497 a	0	0	0	0
Dihydromyricetin-*O*-galloyl hexosoide (18.3)	0.009 ± 0.009 a	0	0	1.958 ± 1.955 a	0
Quercetin-*O*-galloyl hexoside (22.9)	0.001 ± 0.001 a	0	0	8.125 ± 8.11 a	0
Myricetin-*O*-rhamnoside (23.6)	0.067 ± 0.061 a	0.198 ± 0.197 a	2.684 ± 1.581 a	211.43 ± 70.467 b	5.501 ± 3.223 a

Unknown phenolic compound 1 (25.0)	1.184 ± 0.277 a	0.861 ± 0.531 a	0.642 ± 0.403 a	38.518 ± 12.9456 b	2.612 ± 2.022 a
Quercetin (26.6)	2.485 ± 1.232 a	4.044 ± 0.901 a	38.783 ± 19.01 b	0	0
Unknown phenolic compound 2 (27.6)	0	0	0	3.144 ± 3.139	0
Quercetin-*O*-pentoside (28.6)	0.0.178 ± 0.162 a	0.435 ± 0.162 a	1.792 ± 0.434 a	37.665 ± 6.768 b	0
Quercetin-*O*-rhamnoside (29.2)	2.139 ± 0.523 a	3.501 ± 0.331 a	5.339 ± 1.855 a	223.789 ± 80.68 b	14.68 ± 7.618 a
Total water-soluble phenolics	15.189 ± 0.395 a	13.875 ± 0.293 a	199.884 ± 4.623 a	1466.241 ± 33.778 b	69.292 ± 1.559 a