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Effects of Inorganic Mulches on *Achillea millefolium* L. Sesquiterpene Lactones

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Authors' contributions

This work was carried out in collaboration between all authors. Author MPG managed the sesquiterpene lactones study, performed the statistical analysis, and wrote the first draft of the manuscript. Author RMB collected and organized the field data, and author RRF designed the field study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Sesquiterpene lactones are one of the most important groups of bioactive substances in *Achillea millefolium*, a perennial herb cultivated because of its medicinal interest. We sought to determine if the use of inorganic mulches for weed control in *A. millefolium* crops could lead to differences in their sesquiterpene lactones (SLs) content.
Study Design: A field experiment using a randomized complete block design with three replications was conducted to determine the effect of black polypropylene, black polyethylene of 100 μm and black polyethylene of 175 μm on *A. millefolium* SLs content.
Place and Duration of Study: Department of Crop Production, University of Santiago de Compostela, between September 2004 and August 2005.
Methodology: LC/MS analysis of *A. millefolium* flower heads grown over the three different inorganic mulches and in control plots was carried out in order to determine SLs.
Results: Chromatographic analysis of *A. millefolium* flower heads grown over polypropylene and in control plots revealed the highest diversity of SLs, whereas those developed over black polyethylene showed the least. Polypropylene mulches were shown to be the most efficient with a 2-3 fold increase of total SLs (P < 0.001). The effects of inorganic mulches on individual SLs were of limited relevance. However, the use of black polyethylene (100 μm) resulted in an increase of 8-desacetylmatricarin (P = 0.005), a SL of biological and pharmacological interest. In contrast, concentrations of three unidentified

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SLs decreased in yarrow plants grown over black polyethylene.
Conclusion: Weed density did not show as a major factor influencing the total amount of SLs but growth parameters such as yarrow dry matter were inversely related.

Keywords: Black polyethylene; black polypropylene; secondary metabolism; weed density; yarrow production.

1. INTRODUCTION

Achillea millefolium L., also known as common yarrow (Asteraceae) is a perennial herb of medicinal and ecological interest. Phytochemical investigation of *Achillea* species revealed that sesquiterpene lactones (SLs) are the major pool of secondary compounds [1] responsible for such qualities [2,3,4]. Common yarrow is also used in cover-crop systems because of its potential beneficial effects on natural enemies, creating an alternative within integrated weed management plans. Such ecological importance is due to its secondary metabolite profile, which has the capacity to decrease the need for the use of chemicals against pests through promoting an increase of natural enemies, thus providing fortuitous control and decreasing pest pressure in the crops [5,6,7].

Since secondary metabolites display interactions between plants and environment, variations in their total content and/or of their relative proportions are often influenced by environmental conditions. Despite the fact that interactions between plant secondary metabolism and biotic factors are well documented, including a wide range of studies regarding SLs potential for biological control, little information is available on how abiotic conditions affect SLs. External factors, such as light, temperature, humidity and soil fertility, as well as season are proven to have an impact on the secondary metabolism in higher plants [8,9,10,11,12]. Such effects can influence the quality and properties of wild and cultivated plants used in pharmaceutical, nutritional and industrial applications. This is of great importance, since optimizing plant production and the yield of active chemical constituents becomes a priority in medicinal crops.

One way to optimize plant production is to protect crops from weed competition by using mulches of different kinds. Inorganic mulches such as polypropylene and black polyethylene are successful in suppressing weeds and enhancing crops growth and development by reducing moisture evaporation from the soil, increasing soil and air temperature in the plant proximity and decreasing competition [13,14]. Plastic mulches also influence the concentration of valuable chemicals such as terpenoids and phenolic compounds [15,16,17].

Despite some information available on the changes in the concentration of secondary metabolites due to the use of mulching in the crops, these data are mostly referred to phenolic or aroma compounds. No recent study has been published on the influence that environmental changes produced by the use of inorganic mulches might have on SLs.

The aim of our study was to report the variability of sesquiterpene lactones in *A. millefolium* crops grown over black polyethylene and polypropylene mulches. To address this question flower heads of common yarrow grown over these inorganic mulches were investigated. Different genera of the Asteraceae showed that flower heads are best suited for quantitative comparative phytochemical investigations because flower heads are the least affected by seasonal variations of secondary metabolite contents [18]. Data on *A. millefolium* plant growth and weed density in the crops are also reported to discuss possible relationships or influences on the concentrations of SLs.

2. MATERIALS AND METHODS

2.1 Experimental Design and Plant Material

The field experiment was established in San Fiz, Lugo (NW Spain, 43º14' N, 7º28' W, elevation 419 m) in 2005. The average maximum and minimum annual temperatures during the year of the study corresponded to 17.7ºC and 5ºC, respectively, and precipitation was 623mm.Twelve raised beds (i.e. plots), 4m x 2m by 25 cm high each, were prepared at 50 cm intervals comprising a total area of 148 m2 on flat uniform soil conditions. Four treatments were applied for weed control and laid out in a completely randomized design with three replications: black polypropylene anti-weed net (PAWN), black polyethylene 100µm thickness (P100), black polyethylene 175µm thickness (P175), and control (hand-weeding once per month).

Plant material consisted of flowering plants of 5-6 leaves previously produced in September 2004 on a greenhouse bench from seeds with origin in Germany and purchased from Semillas Montaraz S.A. On March 2005 plants were selected for uniformity and transplanted to the field plots through holes that were cut 30 cm x 50 cm apart (36 plants per plot).

Yarrow aerial parts were harvested during the peak of the flowering season (July 2005) in each plot. Once harvested, plant material was separated into fractions (leaves, stems and flowers) and then oven dried at 30ºC. Flower heads for analysis were combined in a single sample per plot. For each treatment three batches of flower heads were collected and analyzed separately, thus a total number of 12 samples were analyzed.

2.2 Extract Preparation

Prior to extraction, a subsample of each batch of oven dried flower heads was ground in a Wiley mill to pass a 20 mesh screen. Yarrow SLs were extracted following a method similar to Avula et al. [19]. Dry ground plant samples (500 mg) were placed in 2.5 ml of methanol for 20 min at 25ºC followed by centrifugation for 15 min at 3,500 rpm. The supernatant was transferred to a 10 ml volumetric flask. The procedure was repeated three times and respective supernatants combined. The final volume was adjusted to volume with methanol and mixed thoroughly. A volume of 3 ml was passed through a 0.45µm Nylon membrane filter. The first 1.5 ml was discarded and the remaining volume was collected in a Liquid Chromatography vial. Previously to the yarrow harvest, density of weeds, and their total dry weight (during May and June) were estimated for each treatment.

2.3 Analysis of Compounds

For the SLs determination we used LC-MS following Montsko et al. [20]. The LC system consisted of an API 4000 LC/MS/MS SYSTEM coupled with an Agilent HPLC 1100 Series. A KINETEX C18 column (2.1 x100 mm, 2.6 µm particle size, 100 A) was used as stationary phase and temperature maintained at 25ºC. A multistep gradient method was applied using acetonitrile- water (2:98, v/v) with 1 mmol ammonium-acetate mixture as solvent A and acetonitrile-water (95:5, v/v) with 1 mmol ammonium-acetate mixture as solvent B at a flow rate of 0.2 ml/min. The gradient profile was 0.0–20.0 min from 0 to 32% B; 20.0–20.1 min from 32 to 100%; 20.1–25.0 min 100% B; 25.0–25.5 min from 100 to 0% B; 25.5–30.0 min 0% B; and 30.0-60.0 min 0% B. Two µl of each sample solution were injected in triplicate with a blank between each triplicate. Santonin (Sigma-Aldrich, 99% purity) as external standard was used at the beginning and at the end of the series (0.1 g santonin in 100 ml methanol). Each peak of the chromatogram was quantified with a seven point standard curve derived from santonin standard solutions, each representing a different concentration level. Concentrations of compounds were expressed in mg of santonin eq/g dry weight.

Mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI). The auxiliary and the curtain gas were nitrogen at 30 psig and 15 psig respectively. The probe temperature was 400ºC, the corona voltage was set at 3 kV. Spectra were recorded in positive ion mode by 0.5 scan/s between m/z 100 and 400. SLs were characterized by MS existing data of sesquiterpene lactones in *A. millefolium* [20, 21] by means of a post-process that comprised the search of the main ions through XIC (the Extracted Ion Chromatogram).

2.4 Data Analysis

Concentrations of each compound and yarrow production were subjected to a one way ANOVA by using STATGRAPHICS Plus (Version 5.0) to determine treatment effects. The normality, homogeneity of variances and possible outliers were checked for each data set by plotting the residuals. On those cases that were required, natural log (Ln) transformations were used to obtain constant variance. In order to know which means were significantly different the ANOVA was followed by a comparison of means using the Fisher's least significant difference (LSD).

3. RESULTS AND DISCUSSION

3.1 Characterization of Sesquiterpene Lactones

Chromatographic analysis of the *A. millefolium* flower head extracts revealed between 20 and 28 major components. Flower heads grown over polypropylene anti-weed net (PAWN) and in control plots (C) showed an average of 25 major compounds, whereas those grown over black polyethylene (P100 and P175) displayed the lowest diversity (20 components). Sesquiterpene lactones were evident on the chromatograms but identification was not possible for all of them due to the lack of authentic reference compounds. The analysis of the mass spectrum and the tentative molecular ions for the unidentified peaks suggested molecular weights within the range of SLs. Those peaks were numbered from SL1 to SL28 with increasing retention times in the chromatograms. The tentative identification by HPLC-MS allowed the characterization of four peaks previously identified as known SLs in *Achillea* species [20, 21]. Two as matricarin-type guaianolides, matricarin ($[M+H]^+$, m/z 305.1), and 8-desacetylmatricarin ($[M+H]^+$, m/z 263.2; $[M+NH4]^+$, m/z 280.1), and two as the eudesmanolides tauremisin ($[M+H]^+$, m/z 265.2; $[M+NH4]^+$, m/z 282.1), and 4-hydroperoxy-arglanin ($[M+H]^+$, m/z 279.1; $[M+NH4]^+$, m/z 296). Tauremisin has cardiotonic properties [22] and matricarin-based SLs have been reported as biologically active natural products [23]. Anti-inflamatory, anti-allergic, and also insect deterrent activities were reported for 8-desacetylmatricarin [2,24,25].

3.2 Influence of Inorganic Mulches on Individual and Total Sesquiterpene Lactones

Levels of five SLs varied significantly among flower heads grown over different types of mulch (Table 1). In black polyethylene, SL10 and SL18 decreased by 12.5-14% and by 32%, respectively, compared with PAWN and control plots. In contrast, levels of SL23 and 8-desacetylmatricarin (SL25) were the highest over P100.

Table 1. Concentrations of sesquiterpene lactones (Mean Values ± SE) from *A. millefolium* flower heads grown over inorganic mulches. Means (n = 3) are miligrams of santonin equivalents per gram of dry weight [a]

Compound	Control	PAWN	P100	P175	*P*- value[b]
Matricarin	0.321±0.016 (32.89)	0.319 ± 0.022 (36.37)	0.330 ± 0.014 (42.37)	0.321 ± 0.007 (44.30)	0.96
4-Hydroperoxy-arglanin	0.077 ± 0.016 (9.54)	0.044 ± 0.016 (5.14)	0.035 ± 0.013 (5.08)	0.021 ± 0.007 (3.92)	0.09
8-Desacetyl-matricarin	0.021 ± 0.003^{b} (2.20)	0.022 ± 0.001^{b} (2.66)	**0.034** ± 0.002^{a} (4.40)	0.023 ± 0.001^{b} (3.24)	0.005**
Tauremisin	0.042 ± 0.003 (4.36)	0.036 ± 0.004 (4.23)	0.046 ± 0.004 (5.90)	0.031 ± 0.002 (4.39)	0.07
SL6	**0.014** ± 0.001^{a} (1.49)	0.011 ± 0.001^{a} (1.34)	0.012 ± 0.001^{a} (1.64)	0.008 ± 0.001^{b} (1.19)	0.02*
SL10	0.007 ±0.002^{a} (0.74)	**0.008** ± 0.002^{a} (1.02)	0.001 ± 0.001^{b} (0.12)	0.001 ± 0.001^{b} (0.24)	0.01*
SL18	**0.022** ± 0.002^{a} (2.30)	**0.022** ±0.006^{a} (2.52)	0.007 ± 0.002^{b} (0.97)	0.007 ± 0.002^{b} (1.00)	0.01*
SL23	0.008 ± 0.000^{ca} (0.87)	0.006 ± 0.001^{bc} (0.74)	**0.010** ± 0.001^{a} (1.31)	0.005 ± 0.001^{b} (0.73)	0.003**

*[a] Only compounds for which significant effects were detected, as well as those identified, are shown. Within each line, mean values followed by the same letter are not significantly different according to F-test. [b] Significances were declared at **, P < 0.01; *, P < 0.05. Relative amount of each compound (% of total) is presented in parenthesis. The highest concentration for each compound is shown in bold.*

The observed influence of mulches on individual SLs might be of limited relevance as it occurred in a few number of SLs (SL6, SL10, SL18, SL23) which are in the medium-low range of relative percentage with respect to the total (Fig. 1).

On the contrary, the concentrations of matricarin (SL1), 4-hydroperoxy-arglanin (SL21), and tauremisin (SL27), compounds of pharmacological interest, were not different in flower heads grown in the various treatments compared to the control. This is relevant as these SLs appeared in higher relative percentages with respect to total, and their sum comprised between 46% and 53% of the total depending on the treatment (Fig. 1).

Because the yarrow crops used in this study produced relatively low levels of individual SLs compared to other *Achillea* species [21], and only five SLs were influenced by treatments, we performed ANOVA for total SLs to gain insight into how their over-all levels were influenced by the inorganic mulches. As was the case with SL10 and SL18, yarrow flower heads developed over PAWN contained the highest levels of total SLs (P < 0.001) with a 2-3 fold increase compared to yarrow cultivated over black polyethylene or in the control plot (Fig. 2).

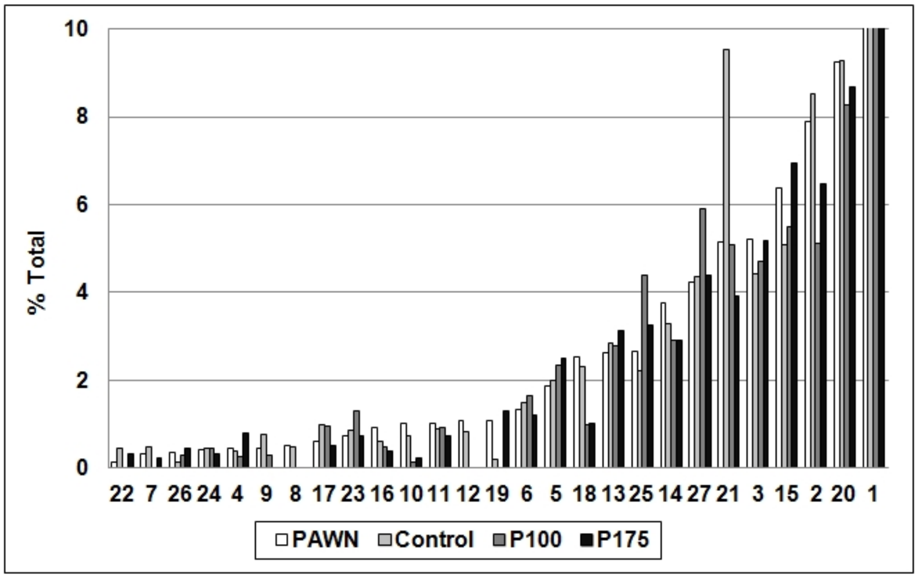

Fig. 1. Individual sesquiterpene lactones (percentage of total) from *A. millefolium* flower heads grown over inorganic mulches (PAWN, black polypropylene; P100, P175, black polyethylene of 100 and 175 µm). Sesquiterpene lactones are labeled based on their retention time in the HPLC analysis

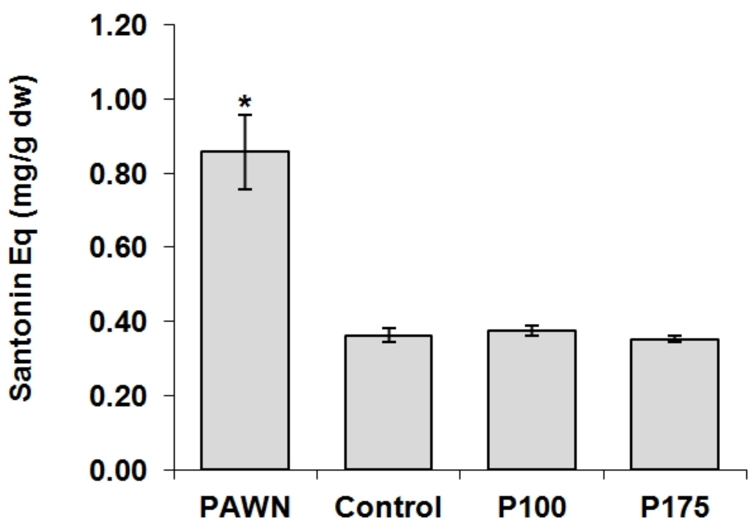

Fig. 2. Total sesquiterpene lactones from *A. millefolium* flower heads grown over inorganic mulches (PAWN, black polypropylene; P100, P175, black polyethylene of 100 and 175 µm). The bars denote means ± SE. * Mean values are significantly different from control (*P* < 0.05)

3.3 Influence of Inorganic Mulches on Growth Parameters

Growth characteristics of yarrow plants expressed as dry weight, plant height, and number of stems per plant were also significantly influenced by inorganic mulches. Opposed to the highest content of total SLs, yarrow plants developed over PAWN accumulated about 17% lower total dry weights (P = 0.048) compared with plants grown either in the control plots or over black polyethylene (Fig. 3).

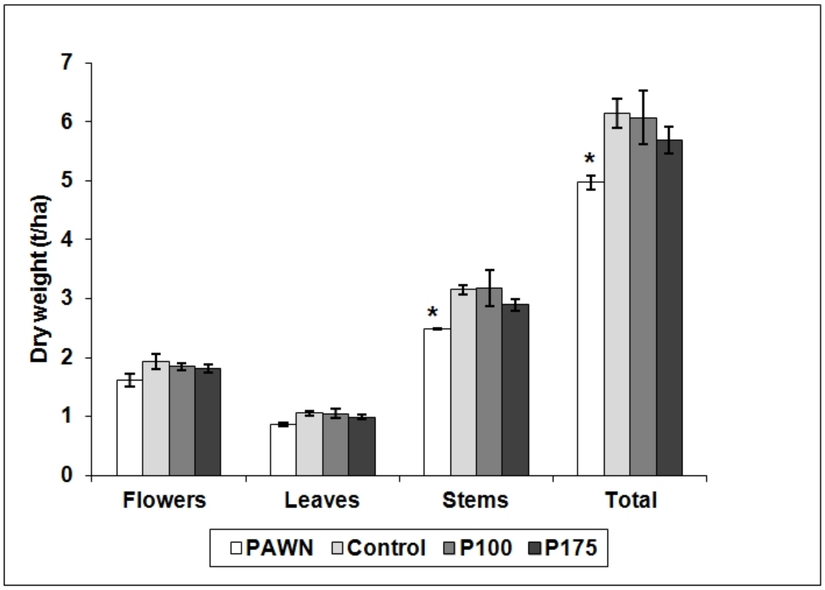

Fig. 3. Influence of inorganic mulches (PAWN, black polypropylene; P100, P175, black polyethylene of 100 and 175 µm) on *A. millefolium* production. The bars denote mean ±SE. * Mean values are significantly different from control (P < 0.05)

Factors other than only the competition of weeds over the crop influenced total yarrow production. This was evident given the lack of differences found between total dry weight of yarrow plants grown over black polyethylene and those located in the control plots which had the highest weed density (Table 2). Black polyethylene was postulated as the most efficient weed control system compared to organic mulches or polypropylene [13], although this was a benefit only in situations where weed control is the primary concern as its lack of porosity has been associated with reduced plant growth.

Table 2. Influence of inorganic mulches on weed density (individuals/m^2) and weed production (g dry weight/m^2) in *A. millefolium* crops[a] (Mean values ±SE)

Treatment	Weed Density		Weed Production	
	May	June	May	June
Control	158.58	156.92	15.67	12.72
PAWN	1.27	15.82	0.13	1.29
P100	0.53	6.65	0.05	0.54
P175	0.53	6.52	0.05	0.54

[a] *Data correspond to weeds existing before yarrow plants were harvested in July*

On the other hand, the use and type of mulch influenced yarrow growth patterns. The significantly lower height (P = 0.02) observed in plants grown in the control plots was compensated by their highest (P < 0.001) number of stems (Table 3). Height/stems ratio found its highest value (P < 0.001) in plants grown over PAWN and P175.

Table 3. Influence of inorganic mulches on yarrow growth[a] (Mean Values ± SE)

Treatment	Plant height (cm)	Number of stems	Height/Stems
Control	60.64 ± 1.54^{b}	34.46 ± 1.25^{a}	1.76 ± 0.02^{c}
PAWN	68.72 ± 2.98^{a}	16.57 ± 1.59^{c}	4.21 ± 0.37^{a}
P100	67.25 ± 0.99^{a}	22.48 ± 1.15^{b}	3.00 ± 0.13^{b}
P175	70.28 ± 0.08^{a}	16.07 ± 1.22^{c}	4.42 ± 0.35^{a}

[a] Expressed as plant height and number of stems per plant. Within each column, values followed by the same letter are not significantly different (P > 0.05).

Some studies have postulated that the benefits of inorganic mulches on the crop growth do not relate with an increase of its active chemical constituents [15,26]. This negative correlation has been often discussed in terms of a physiological trade-off between plant growth and secondary metabolism, since the latter represents a cost to the plant. Because many documented studies confirm that the production of secondary metabolites should vary as a result of a trade-off with primary biological functions, or as a response to biotic and abiotic environmental parameters [27,28], we hypothesize that the inverse relationship found between total SLs content in yarrow flower heads and total yarrow production could possibly be related to the ecological hypothesis regarding interactions such as the Growth-Differentiation Balance Hypothesis. This hypothesis states that there is a physiological trade-off between growth and secondary metabolism imposed by developmental constraints in growing cells, and competition between primary and secondary metabolic pathways in mature cells [27,29].

Additional research would be interesting to determine the potential of *A. millefolium* as one possibility for using allelopathy to improve the overall potential of weeds and crops in natural weed management. SLs can have allelopathic effects on crops and many weeds are now achieving importance as an agent of weed control for having special types of allelochemicals [30].

4. CONCLUSION

The main result of the current study is the pronounced increase of total SLs with the use of polypropylene mulches in *A. millefolium* crops. Effectiveness of black polyethylene on individual SLs yield differed and was very limited, with neutral or negative effects in general for P175. Only a slight advantage was found in yarrow crops protected by P100 because of their significantly higher 8-desacetylmatricarin levels, a SL of biological and pharmacological interest.

Weed control performed by the inorganic mulches in this study was neither directly correlated with yarrow production nor with total SLs yield, which might suggest that additional factors, other than competition of weeds over the crop exclusively, are influencing yarrow production as well as concentrations of SLs in yarrow flower heads. Common yarrow grows well in a wide range of habitats and presents an inherent rusticity which makes it difficult to predict the impact of an individual environmental factor and to provide a general explanation of the observed effects.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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