

## CHAPTER III – PRODUCTION DYNAMICS OF PARTHENIN IN THE LEAVES OF *PARTHENIUM HYSTEROPHORUS*

### 3.1 Introduction

Parthenin, a sesquiterpene lactone, is believed to play a major role in the allelopathy of *P. hysterophorus*, and it may play a role in the displacement of naturally occurring vegetation for the weed to become established in an area. On the molecular level, sesquiterpene lactone biosynthesis is regulated at the transcriptional level, and these compounds generally originate from the mevalonic acid pathway (Duke & Inderjit, 2003). It has been suggested that all terpenes originate from the common precursor, isopentenyl diphosphate (Fonseca *et al.*, 2005). In addition to its phytotoxic properties, parthenin is also known for its allergenic, anti-feedant and anti-microbial properties. Parthenin has been reported to be located in various plant parts with especially high concentrations occurring in trichomes on the leaves (Kanchan, 1975; Towers *et al.*, 1992; McFadyen, 1995; Reinhardt *et al.*, 2004). Four types of glandular and non-glandular trichomes occurring on the leaves and achene-complex were described by Rodriguez *et al.* (1975) who identified parthenin and ambrosin in external chloroform washings of flowers and leaves. Reinhardt *et al.* (2004) determined that one trichome type in particular, the capitate-sessile trichome, contained virtually 100% parthenin. Reinhardt *et al.* (2004) further quantified the amount of parthenin present in one capitate-sessile gland at 0.3 µg parthenin per gland and suggested that these trichomes are the main source of parthenin that is released from the plant. Furthermore, they proposed that extrapolation of per plant parthenin amounts to field-scale production makes it plausible that parthenin can contribute significantly to the ability of *P. hysterophorus* to displace other species.

Allelochemical production in living plants is apparently affected by biotic and abiotic factors (Dakshini *et al.*, 1999), which in turn affect a plant's allelopathic potential (Hedin, 1990; Lovett & Houtt, 1995; Einhellig, 1995). Periodic peaks in allelochemical production have been reported, especially in response to biotic factors (Woodhead, 1981; Baldwin, 1989). The production of secondary metabolites is determined by a plant's genetic make-up in combination with environmental factors

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(An *et al.*, 2003). Stressful environmental conditions, such as abnormal radiation, mineral deficiencies, water deficits, temperature extremes, and pathogen/predator attack can induce increased allelochemical production in plants (An *et al.*, 2003). This can be beneficial in several ways. Phenolics are considered to protect plants from UV radiation (McClure, 1975), and allelochemicals may advantage the producer under stressful conditions that result in resource competition (Kuo *et al.*, 1989), plus these compounds can protect against pathogens and herbivores (Picman *et al.*, 1981; Datta & Saxena, 2001). The decrease with age of allelochemical concentrations in living plants has been well documented in several instances (An *et al.*, 2003), but there are exceptions (Woodhead, 1981). Chou (1999) suggested that allelochemicals possibly perform an autotoxic role in order to regulate population levels according to growth conditions and resource availability.

An obvious advantage for attaining and maintaining dominance in a plant community would be sustained production of allelochemicals at high levels throughout the life cycle of a plant. Increased production towards the end of a life cycle could point to a strategy of reliance on allelopathic residues for suppressing the germination and establishment of other, or even the same, species. Considering the location of parthenin in *P. hysterophorus* (Reinhardt *et al.*, 2004) it is most likely that parthenin is released either through leaching off leaves and/or in the process of leaf decomposition. The combined process of parthenin production, release mechanism(s), and its persistence in the environment will determine its own contribution to the overall allelopathic effect of *P. hysterophorus*. However, growth responses of acceptor plants will not be determined only by the parthenin effect, but also by that of other allelochemicals produced and released by *P. hysterophorus*. The relative contribution of the various allelochemicals associated with *P. hysterophorus* to its allelopathic influence is still not fully understood (Belz *et al.*, 2006), but clearly there is much evidence to suggest that parthenin plays a major role.

Little is known about the production and release of parthenin during the growth stages of *P. hysterophorus*. Earlier, Belz *et al.* (2006) observed variability in the amounts of parthenin extracted from the leaves of the same plants harvested at different stages, and speculated that these differences may be age-dependent. The aim of the current study was to investigate parthenin production dynamics by determining parthenin

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concentrations in parthenium leaf material at different phenological stages of the plant. This will contribute to further illumination of the role of parthenin in *P. hysterophorus* allelopathy.

### 3.2 Materials and methods

#### 3.2.1 Cultivation and harvesting of *P. hysterophorus* plants

*P. hysterophorus* plants were cultivated under greenhouse conditions (13/11 h, 22/18 °C, 300  $\mu\text{E}/\text{m}^2\text{s}$ ) at the University of Hohenheim. Plants were grown from seed collected at an infested site in Kruger National Park, South Africa. Seeds were pre-germinated in vermiculite and 25 days later seedlings were transplanted into pots (15 x 15 x 20 cm) filled with a 1:3 (v/v) mixture of humus soil (Humusoil, Floragard, Germany) and sand as growth medium. Watering was done as required with tap water and fertilizer was applied once weekly [1 ml L<sup>-1</sup> Wuxal® Super (Fa. Aglukon Spezialdünger, Germany)]. Plants were harvested at different phenological stages from the 4-leaf stage until senescence. Parameters measured at each harvesting included: total number of leaves, fresh and dry mass of leaves, fresh mass of entire plant, and plant height (from base to tip of uppermost leaf). Fresh leaf material was frozen at -20°C immediately after harvesting for chemical analysis of parthenin.

#### 3.2.2 Chemical analysis

##### 3.2.2.1 Sample preparation

Frozen samples were defrosted and diced into sections of 1 cm<sup>2</sup>. As the moisture percentage of leaf material harvested at different stages would vary, a portion of the leaf material was used to measure the dry weight and determine moisture percentage of the sample. Depending on the amount of leaf material available for analysis, 0.4 - 12 g of leaf fresh weight was analyzed per replicate. A mixture of acetonitrile:water [1:1 (v/v); ACN:H<sub>2</sub>O] was added to the leaf material at a concentration of 0.1 g ml<sup>-1</sup>. The chopped leaf material together with ACN:H<sub>2</sub>O was homogenized for three minutes at 20 000 rpm with an Ultra Turrax blender (Janke & Kunkel Ltd.,

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Germany). The homogenate was filtered, centrifuged (10 min; 20 000 rpm), and a 1 ml aliquot was transferred to a glass vial for chemical analysis.

### 3.2.2.2 Preparation of pure parthenin standard

Preparative high-performance liquid chromatography (HPLC) was used to obtain parthenin as HPLC standards [as described by Belz *et al.*, (2006)]. Fresh leaf material from *P. hysterophorus* plants was dipped for ten seconds in tert-butyl methyl ether (250 mg FM ml<sup>-1</sup> TBME). Organic leaf extracts were filtered over anhydrous sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) and the extract concentrated with a rotary evaporator (40°C, 250 mbar). The oily, green residue obtained was re-dissolved in 1:1 (v/v) ACN:H<sub>2</sub>O and fractionated by preparative HPLC (Varian model chromatograph) with UV detection (Varian UV-VIS detector model 345; detection wavelength 225/254 nm). A Grom Nucleosil 120 C-4 column [250 mm by 16 mm (5 µm), Grom, Germany] was used, and eluted with a gradient of 20% ACN and 80% Na<sub>2</sub>HPO<sub>4</sub>-buffer (1 mM, pH 3, 10% ACN) for 0-20 min, 100% ACN for 20-26 min, then re-equilibrated to starting conditions (6 ml min<sup>-1</sup> flow rate). Injection volume was 100 µl. Parthenin was identified in the fraction ranging from 9.1 – 10.3 minutes. Standard purity was verified by HPLC-DAD and results confirmed by HPLC-ESI-MS.

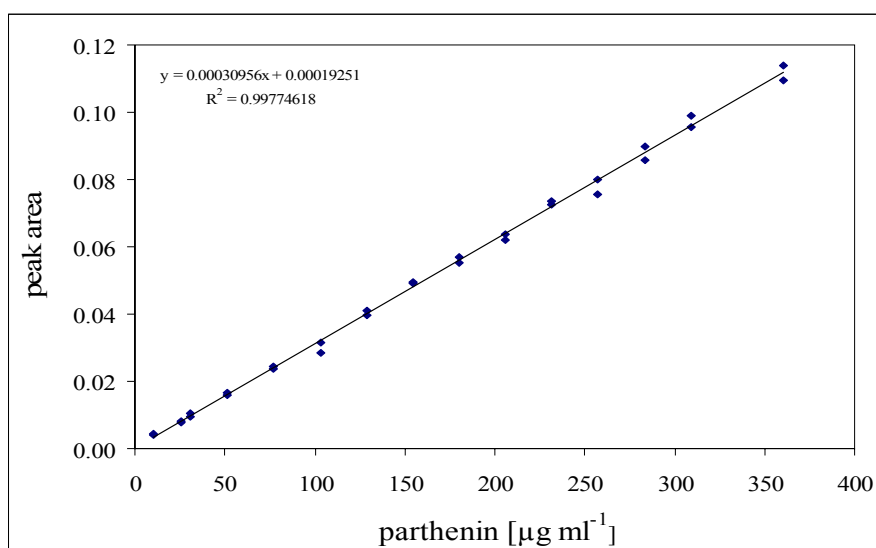
### 3.2.2.3 Quantification of leaf parthenin content

HPLC analysis (Waters model chromatograph) with DAD detection (photodiode array detector, Waters 991) for determination of parthenin in leaves was done according to Belz *et al.* (2005). A Synergi polar C-18 reversed phase column [250 mm by 4.6 mm (4 µm), Phenomenex, Germany; 35°C column oven temperature] was used, and eluted with a gradient of 5% ACN and 95% Na<sub>2</sub>HPO<sub>4</sub>-buffer (1 mM, pH 2.4, 10% ACN) for 0-8 min (0.65 ml min<sup>-1</sup> flow rate), 30% ACN and 70% Na<sub>2</sub>HPO<sub>4</sub>-buffer for 8-26 min (0.7 ml min<sup>-1</sup> flow rate), 100% ACN for 26-29 min (0.7 ml min<sup>-1</sup> flow rate), 100% ACN for 29-31 min (0.7 ml min<sup>-1</sup> flow rate), then re-equilibrated to starting conditions. Injection volume was 50 µl. Parthenin was identified and quantified at 220 nm. Retention time was 26.07 ± 0.02 min. Quantitative analysis was done by external calibration curves.

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## 3.2.2.4 Calculation of parthenin concentration

A clear peak was observed on the HPLC chromatogram at 17.25 min and was identified as parthenin. Pure parthenin standards with known concentrations were used to obtain a parthenin concentration versus peak area calibration line (Figure 3.1). Parthenin concentration in the samples could then be calculated using the computer generated equation for this line.



**Figure 3.1** Parthenin concentration versus peak area calibration line

Parthenin concentration of the leaf extracts was calculated using the following equation:

$$\frac{x \mu\text{g/ml} ([\text{Sample}]) \times z \text{ ml (ml of extract)}}{\text{g (initial weight)}} = \mu\text{g parthenin/g initial weight}$$

## 3.2.3 Statistical analysis

Parthenin concentrations in extracts prepared from leaves of plants harvested at the same growth stages were analyzed using SAS® to detect significant differences. Data were analyzed after a logarithm transformation to achieve normal distribution of the data. A general linear model (GLM) of ANOVA was used and significant differences between means were determined using Tukey's studentised range test at  $P \leq 0.05$ .

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**3.3 Results and discussion**

Mean leaf moisture percentage was observed to decrease with plant age (Table 3.1).

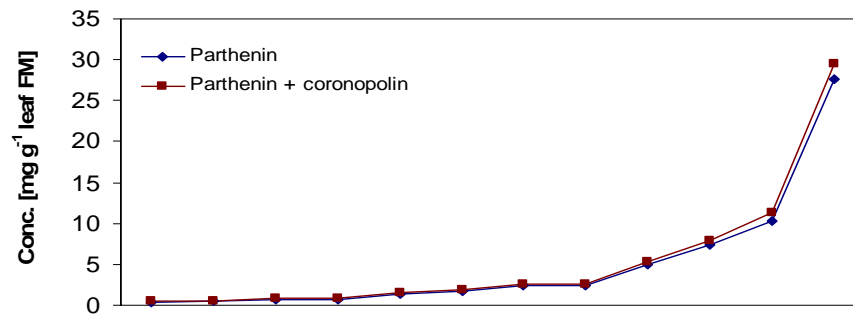
**Table 3.1** Mean leaf moisture percentages at different growth stages

Growth stage	Mean water content [% of FM]
10-51	86.1 ± 4.2
41-60	81.9 ± 4.6
70	64.8 ± 7.6
80	20.3 ± 0

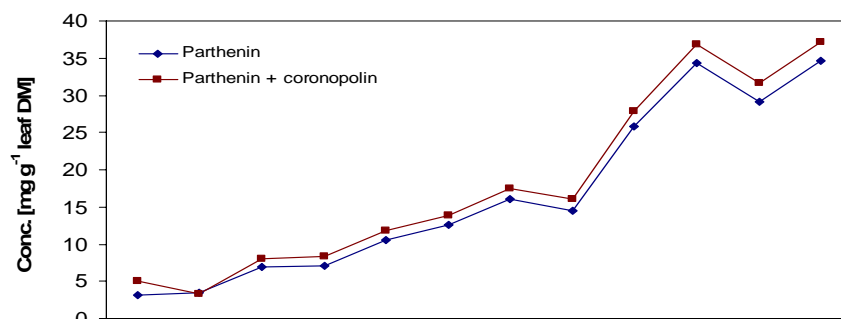
Growth stages: 10-51: Beginning of leaf development to flowering in all leaf axils; 41-60: Flower buds formed in all axils to fruit development; 70: Ripening/maturity of fruit and seeds; 80: Senescence.

An increase in parthenin concentration with plant age was observed (Figure 3.2), with highest levels occurring in the final three growth stages for both fresh and dry mass, as well as for overall parthenin content in all leaf material.

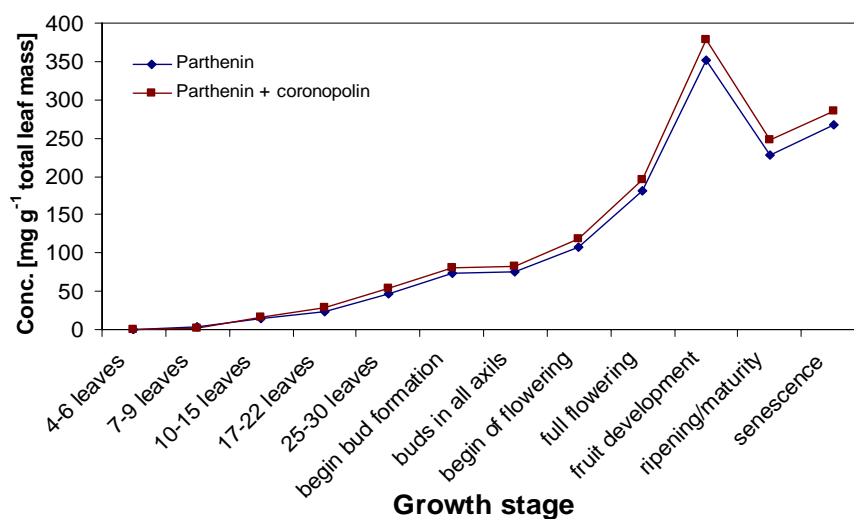
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(a)



(b)



(c)

**Figure 3.2** Concentrations of parthenin as well as parthenin and coronopalin in leaf fresh (a) and dry (b) material at different growth stages of the plant according to the BBCH code; and (c) total parthenin content in plant leaf material at the different growth stages

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Highly significant differences were observed for parthenin concentration at different phenological stages (Table 3.2). Youngest leaves produced the least parthenin, and oldest leaves the most. Highest parthenin concentrations occurred in the final three growth stages under the experimental growth conditions. The parthenin analogue, coronopolin, which is considered to be biologically inactive, was also analysed and found to follow closely the production trend of the former over the entire life-cycle of the plant (Figure 3.2).

**Table 3.2** Parthenin concentrations in leaf dry mass of plants at different growth stages (Appendix 3.1)

<b>Growth stage</b>	<b>Mean water content [% of FM]</b>	<b>Total number of leaves</b>	<b>Total FM of leaves [g]</b>	<b>Total DM of leaves [g]</b>	<b>FM of entire plant [g]</b>	<b>Plant height [cm]</b>	<b>Parthenin [mg g<sup>-1</sup> leaf DM]</b>
4-6 leaves	88.6±1.6	5.4	1.3	0.2	1.4	8.3	2.94 d
7-9 leaves	86.0±4.3	7.6	4.2	0.6	4.7	13.1	3.41 d
10-15 leaves	89.7±1.1	11.8	16.8	1.7	19.9	22.5	6.58 dc
17-22 leaves	89.1±1.2	19.0	26.6	2.9	31.6	26.8	6.97 dc
25-30 leaves	87.2±2.6	27.8	35.8	4.6	43.6	27.4	11.10 bc
begin of bud formation	86.5±3.6	27.6	40.5	5.5	53.2	35.6	12.59 abc
buds in all axils	84.9±3.4	40.7	31.6	4.8	50.5	48.0	16.13 abc
begin of flowering	84.8±4.3	44.8	45.0	6.8	67.4	50.3	14.53 abc
full flowering	80.5±3.5	77.3	38.4	7.5	86.0	83.0	25.85 ab
fruit development	77.0±2.6	146.3	48.6	11.2	148.1	124.3	34.33 a
ripening/maturity	64.8±7.5	164.5	23.1	8.1	115.4	112.5	29.15 ab
senescence	20.3±0.0	77.0	9.7	7.7	85.9	125.5	34.7 a

Means followed by different letters differ significantly (Tukey –test, P=0.05)



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Reinhardt *et al.* (2004) reported a parthenin concentration of  $14.5 \text{ mg g}^{-1}$  dry mass in leaves of flowering plants cultivated in a greenhouse at the University of Hohenheim. Parthenium leaf material harvested from flowering plants in Kruger National Park in December 2004 yielded a parthenin concentration of  $16.73 \pm 1.76 \text{ mg g}^{-1}$  dry mass (Belz *et al.*, unpublished). These values correspond with the findings of the present experiment for plants at the bud formation to beginning of flowering growth stages. Kraus (2003) noted that trichome density decreased with leaf expansion and leaf age, and this correlated with higher parthenin concentrations in juvenile leaves. However, when parthenin content of leaf homogenate was analysed, higher parthenin concentrations was found in older leaves. Secondary metabolite chemical concentrations have been found to differ between younger and older leaves (Koeppel *et al.*, 1970; Harrison, 1982). Differences in parthenin content between older and younger leaves of the same plant were not considered in this experiment. Under the conditions that prevailed in the present experiment, parthenin did not decrease with plant age as has been observed for numerous other allelochemicals (Koeppel *et al.*, 1970; Woodhead & Bernays, 1978; Weston *et al.*, 1989; Wolfson & Murdock, 1990).

It may be considered logical that if allelochemicals play a role in plant defence it might mean that the concentration of these allelochemicals could decrease with plant age. (An *et al.*, 2003). A build-up of allelochemicals with age may, however, be important if a plant utilizes residual allelopathy in its interference strategy. Such a strategy would be aimed at avoiding or limiting the recruitment of other, or even the same, species.

High levels of parthenin have also been reported in the flowers and achenes of parthenium (Rodriguez *et al.*, 1975; Picman *et al.*, 1979). Reinhardt *et al.* (2004) measured parthenin concentrations in the flowers and achenes at  $3.7 \text{ mg g}^{-1}$  and  $4.4 \text{ mg g}^{-1}$ , respectively. Parthenin concentrations in achenes from plants grown in the University of Hohenheim glasshouses and from plants growing in the Kruger National Park were measured at  $9.63 \text{ mg g}^{-1}$  and  $28.46 \text{ mg g}^{-1}$ , respectively. These additional sources of parthenin will boost the potential quantity of parthenin that could be released into the environment. At senescence, plants were calculated to contain a final parthenin content of 267.19 mg. Over the life cycle of *P. hysterophorus*, a single plant can therefore introduce  $> 267.19 \text{ mg}$  into the environment in a single growing season.

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The exact mechanism(s) of parthenin release from the plant is still speculative. Rodriguez *et al.* (1975) reported an abundance of trichomes in dry plant parts that had been disseminated by the wind. Kanchan & Jayachandra (1980a, b) observed that the trichomes can easily become detached from dry parts of parthenium and observed leaching from live vegetative parts. Although it is difficult to predict how much parthenin would be released from the plants under natural conditions, it is clear that the plant does retain high levels of parthenin right until the end of its life-cycle. In addition to the role of parthenin in allelopathy, parthenin may also play an important role in herbivore and pathogen defence. Maintaining high parthenin levels in the plant until after flowering may therefore be of huge benefit to the plant.

Duke *et al.* (2000) points out that ‘few systematic studies exist of how cultural methods and the environment affect the production of trichome-borne compounds’. Kimura *et al.* (2000) reported changes in the metabolite level of trichomes in response to environmental changes. Generally, it was observed that allelochemical production increased under stressful conditions for donor plants (Niemeyer, 1988; Putnam, 1988). Fonseca *et al.* (2005) observed changes in the levels of a sesquiterpene lactone, parthenolide (PRT) levels in feverfew (*Tanacetum parthenium* L.). PRT levels varied on a daily basis, and increased in plants recovering from water stress. Perhaps even higher levels of parthenin than those found in the present study can be expected in plants growing in natural environments, for example, in the Kruger National Park, where plants are subjected to a range of severe stresses such as intermittent droughts and fire.

Under the trial conditions, parthenin was not observed to decrease with plant age as has been observed to be the case for numerous other allelochemicals studied (Koeppel *et al.*, 1970b; Woodhead & Bernays, 1978; Weston *et al.*, 1989; Wolfson & Murdock, 1990). It can not be assumed, however, that greenhouse conditions are comparable to natural conditions and knowledge of the influence of precipitation, wind and other factors on parthenin release is lacking.

### 3.4 Conclusions

The increase of leaf parthenin concentrations with plant age, and attainment of highest parthenin concentrations in the final three growth stages, indicate a high resource allocation priority of the plant towards this secondary metabolite. This may be indicative of the importance of this compound in the well-being of the plant through allelopathic interactions, pathogen and/or herbivore defence, or in multiple roles. Weidenhamer stated (1996) 'Quantification of allelochemical release rates in the environment and the demonstration that concentrations are sufficient to inhibit growth are key steps in validating a hypothesis of allelopathic interference'. Further research in this direction should study the influence of abiotic and biotic factors on parthenin production, and the modes of parthenin release from the plant.

(Note: The findings presented in this chapter have since been published: Reinhardt *et al.*, 2006).