Modelling Processes Determining and Limiting the Production of Secondary Metabolites during Crop Growth: the Example of the Antimalarial Artemisinin Produced in *Artemisia annua*

W.J.M. Lommen[1]*, H.J. Bouwmeester[2], E. Schenk[1], F.W.A. Verstappen[2], S. Elzinga[1] and P.C. Struik[1]
[1] Crop and Weed Ecology Group, Wageningen University, Wageningen, The Netherlands
[2] Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands
* Corresponding author

Keywords: malaria, medicinal plants, modelling, secondary plant compounds, trichomes, yield

Abstract

The quantitative insight in processes underlying yield and concentrations of interesting secondary metabolites in crops is still limited. Yet, this insight is essential to further improve crops and commercial production of target metabolites. *Artemisia annua* L. (annual or sweet wormwood, Asteraceae) was used to conceptualize a model to describe the processes determining and limiting the production of target metabolites during crop growth. *A. annua* is an annual herb producing the antimalarial artemisinin, a sesquiterpene lactone with an endoperoxide bridge. Artemisinin is predominantly produced in glandular trichomes present on the leaves and inflorescences. Leaves are the most important organs harvested in commercial production. The accumulation of artemisinin in the crop was analysed as the resultant of the key processes determining leaf dry weight production, accumulation of artemisinin in the leaves and losses after synthesis. A yield formation modelling approach was used to quantify artemisinin yield as a function of the individual processes and to study which processes limited production. Leaf dry weight production was limited by low total dry matter production of the crop because of poor radiation interception during early canopy expansion, and by a high proportion of dry matter allocated to stems that do not contribute to artemisinin production. Production of the target metabolite in the leaves was limited because the total production of artemisinin precursors per unit leaf dry weight was low and because the conversion of precursors to artemisinin was only partial. Possibilities to increase the values of the different yield components are discussed. A new, simple, model for explaining variation in artemisinin yield based on leaf and trichome production in combination with biosynthesis of artemisinin is proposed.

INTRODUCTION

Interest in plant secondary metabolites that have a protective or curative effect on human diseases has been renewed. These compounds can be taken in as drugs or as part of food and beverages. Yields and concentrations of target metabolites in crops or crop products are usually low and highly variable over studies. Also within crops, concentrations vary. The causes of these fluctuations are often unknown because commercial production methods are commonly based on applied dose-effect studies with limited attention to the crop physiology behind the effects. A great need exists for more insight into the different processes underlying the accumulation of the secondary metabolites of interest. An improved understanding can lead to more stable yield levels and concentrations, better breeding strategies, more founded selection of production environments, and agronomical and technological innovations in the production chain.

To achieve a full and quantitative understanding of accumulation of secondary metabolites is difficult because mechanisms underlying their yields and concentrations in crops are more complex than those underlying yield of plant organs, yield of primary

metabolites, or their concentrations. The basic processes playing a role in primary metabolite formation also play a role in the synthesis of secondary metabolites, but more processes are involved: processes affecting the formation of the specialized structures in which the compounds are synthesized (such as ducts or glandular trichomes), processes affecting the production of the compounds of interest and their precursors in these structures, and processes regulating the conversion of the precursors to the target compounds. External factors quantitatively affect these underlying processes through their effects on crop development, growth rates, dry matter partitioning and partitioning of metabolites to the secondary metabolite of interest and these factors can also trigger abrupt activation of qualitative changes in secondary metabolite production.

The Model Crop: *Artemisia annua*

Artemisia annua L. (annual or sweet wormwood, Asteraceae) is an annual herb that produces the antimalarial compound artemisinin, a sesquiterpene lactone that is effective against multidrug resistant strains of *Plasmodium falciparum*. *P. falciparum* causes the severe malaria tropica resulting in more than one million deaths annually. For pharmaceutical use, artemisinin is extracted from the dry leaf mass, purified and chemically derivatised (e.g., Dhingra et al., 2000) and preferably used in combination with other antimalarials.

A. annua is thus far the only economical source of artemisinin. The plant originates from South-east Asia (Ferreira et al., 1997), likely China, but is now widely distributed. The plant is cultivated mainly in Vietnam and China. Within the plant, artemisinin is found almost exclusively in leaves and inflorescences (Ferreira et al., 1995). Leaves are the most important organs for commercial production, as crops are usually harvested before full flowering. Artemisinin is phytotoxic, and production of artemisinin is limited to the very small glandular trichomes on the leaves and inflorescences (Duke and Paul, 1993; Duke et al., 1994). Recently, the biosynthetic route was elucidated for the genotypes used in our study (Bertea et al., 2005).

Agronomic problems in *A. annua* are comparable to those in other crops grown for their secondary metabolites. Yields and concentrations of artemisinin are extremely low. Maximum yields of artemisinin are between 20 and 40 kg per ha. Maximum concentrations of artemisinin in the dry plant leaves are c. 1% (Magalhaes et al., 1996) but usually concentrations are much lower (Morales et al., 1993; Laughlin 1993, 1995). In addition, there is a high variation in yield and concentrations, and almost no insight in what is causing these variations.

In our study on artemisinin production, we want to identify and quantify the processes at the level of the crop, individual organs and cells which are underlying artemisinin formation in commercial *A. annua* crops. The study should lead to (a) a simple model to explain artemisinin production in field crops, and (b) insight in which processes limit artemisinin production during crop growth.

This paper describes the state of the art of this study.

MATERIALS AND METHODS

The ideas for modelling harvestable artemisinin were developed in discussions and by literature study. The experimental work was done using either an open pollinated Vietnamese selection (Bouwmeester et al., 1999; Wallaart et al., 2000; Bertea et al., 2005; Lommen et al., 2006) or the hybrid cultivar Anamed A3 (cf. Lommen et al., 2006), and was carried out at two locations (Achterberg and Wageningen) in The Netherlands; experiments included different planting densities.

The experimental data presented are from one experiment using the Vietnamese selection. Plants were grown from seeds, raised in transplanting trays in the glasshouse and transplanted into the field in Wageningen, The Netherlands (N51°59'31" E005°39'08") on May 21, 2002, 49 days after sowing, at 75 cm between- and 38 cm within-row distance. The plots were part of a larger experiment laid out in five blocks. Net plots were 1.5 x 1.52 m² (2 rows x 4 plants) and were surrounded by at least 1.5 m of

guard rows or 1.52 m of guard plants in a row. Interception of photosynthetically active radiation (PAR) was measured regularly during field growth using the SunScan Canopy Analysis System (Delta-T Devices Ltd, Cambridge, UK). The crop was harvested at 112 days after transplanting into the field. Subsamples for dry matter determination and plant composition were dried at 70°C for a minimum of 24 h. Subsamples for determination of artemisinin and upstream precursors of artemisinin dihydroartemisinic acid, dihydroartemisinic aldehyde, artemisinic aldehyde and artemisinic alcohol were dried at 30°C for 24 h, ground and subjected to GC-MS analysis as described by Lommen et al. (2006).

PROCESSES TO BE MODELLED

The processes underlying artemisinin accumulation in crops were divided into three groups:

Processes Determining the Production of Desired Organs (in *A. annua*: the Leaves)

This group involves processes determining the radiation interception by the crop, the total dry matter production from the intercepted radiation and the partitioning of the produced dry matter to organs contributing (leaves) or not contributing (stems) to the accumulation of the target compound.

Processes Determining the Production of the Target Compounds in These Organs

This group involves processes determining the differentiation of trichomes, the production of precursors and the conversion of precursors to target compounds.

Processes Determining Losses after Formation

This group of processes comprised both losses of whole leaves or leaf material and losses of artemisinin from the leaves.

For the first group of processes (those determining leaf production), the Monteith model of light interception and radiation use efficiency (Monteith, 1977) was adopted to model total above-ground dry matter production and this model was extended to determine dry matter allocation to harvested organs (e.g., Struik and Lommen, 1999). The total model *(1)* explained the yield of the desired organs - in our case the leaves - as a function of:

- the PARsum, the incident radiation over the total growing period - in our case the PAR, the photosynthetically active part of the global radiation;
- the $f_{\text{intercepted PAR}}$, the fraction of the PARsum that was intercepted by the crop (total over the field period);
- the radiation use efficiency (RUE), the efficiency by which the intercepted PAR was converted into dry matter;
- and the harvest index (HI), or the fraction of the total dry matter produced that was allocated to the useful part of the crop, in our case the leaves.

$$\text{Leaf DM yield} = \text{PARsum} \times f_{\text{intercepted PAR}} \times \text{RUE} \times \text{HI} \qquad (1)$$

We extended this approach to deal with the accumulation of artemisinin in these leaves, regarding the total artemisinin production *(2)* to be a function of:

- the leaf dry matter production (Leaf DM yield, the result of formula *(1)*);
- the fraction of precursors + artemisinin in the leaf dry matter ($f_{\text{prec+art in leafDM}}$);
- and the conversion of precursors to artemisinin, i.e., the fraction artemisinin in the total quantity of artemisinin plus precursors ($f_{\text{art in (prec+art)}}$).

$$\text{Artemisinin yield} = \text{Leaf DM yield} \times f_{\text{prec+art in leafDM}} \times f_{\text{art in (prec+art)}} \qquad (2)$$

RESULTS

The crop took almost three months to completely cover the soil and intercept all incoming radiation (Fig. 1). This slow canopy development resulted in a poor radiation interception early in the growing season when radiation levels were relatively high.

From the daily values, the PARsum and the PAR intercepted over the whole growing period were calculated (Table 1). The PARsum during 112 days of field growth was just over 900 MJ/m². Of this incident PAR, a fraction of only 0.504 was intercepted by the crop canopy, leading to 467 MJ PAR/m² intercepted by the crop. This intercepted radiation was converted to above-ground dry matter at an efficiency of 2.5 g dry matter/MJ PAR intercepted, resulting in a dry matter production of 1134 g/m². A fraction of 0.242 of this total dry matter was allocated to the useful organs, the leaves, and the remaining part to the stems. This resulted in a leaf yield of 284 g/m² or almost 3 t/ha. Roots were not included in the analysis.

Only a small fraction of this leaf dry matter consisted of artemisinin and artemisinin precursors, and the total yield of artemisinin and precursors therefore was low (Table 2). From the total weight of artemisinin and assessed precursors, artemisinin comprised 46%, leading to an artemisinin yield of 0.31 g/m² or 3.1 kg/ha (Table 2).

When the relative fraction of artemisinin and precursors was compared on a molar basis, artemisinin contributed 41% and dihydroartemisinic acid, the last artemisinin precursor produced exclusively enzymatically, 43% of all assessed compounds (Fig. 2). The contribution of precursors further upstream of dihydroartemisinic acid was relatively low.

DISCUSSION

The fraction of the incoming PAR intercepted by *Artemisia* crops (c. 0.50 in the given experiment) might be a major limitation for artemisinin production (Table 1, Fig. 3). This fraction of PAR intercepted could probably be increased by crop improvement and cultural practices aiming at advancing canopy growth. An obvious technique to consider would be to increase plant density. This, however, will only lead to improved yields when the positive effect on radiation interception is not compensated for by negative effects on other processes, like a lower fraction of dry matter allocated to leaves.

No limitations in the radiation use efficiency seem to exist as a value of 2.5 g/MJ is comparable with that for other unstressed C3-crops under comparable conditions (Table 1) (Monteith, 1977).

A major yield limiting process was the low fraction of dry matter allocated to the parts of the crop that contribute to the artemisinin production (the leaves) (Table 1, Fig. 3). Most dry matter was allocated to the stems that need to support a crop of over 2 m in height. Improvements could likely be achieved by genotype development and crop management techniques aimed at reducing stem growth. Repeated harvesting or growing *Artemisia* similar to tea crops are management techniques that are being developed (Kumar et al., 2004) and these techniques may increase yield. Growing conditions could also affect the leaf:stem ratio.

The total weight of artemisinin + precursors was only a small fraction of the leaf dry weight (Table 2). As glandular trichomes are the sites of artemisinin production within the leaf, differentiation of trichomes is an essential step for artemisinin production. Genotypes without glandular trichomes do not produce artemisinin (Duke et al., 1994). The relative importance of trichome densities or individual trichome productivity for explaining differences in artemisinin yield over crops is still largely unknown. No experimental data for studies on whole crops is available, but studies on individual leaves within a crop have revealed that trichome densities can partly explain the differences in artemisinin concentrations within a crop (Lommen et al., 2006). Genotype and environmental conditions also likely affect trichome densities. In *Wigandia urens*, for instance, a higher light intensity increased the trichome density (Pérez-Estrada et al., 2000).

Quantitative limitations in production capacity of artemisinin and precursors in the trichomes most probably exist. These limitations are still largely unknown and should be explored in the future. Both environmental conditions and genotype are likely to affect the production capacity. In addition, biotic or abiotic factors could also act as triggers to initiate production of artemisinin and precursors. Differences in production capacity of trichomes are thought to explain part of the differences in artemisinin concentrations observed within plants (Lommen et al., 2006).

Finally, the conversion of precursors into artemisinin would limit artemisinin formation (Fig. 2). The conversion of dihydroartemisinic acid, the last precursor of artemisinin that we assessed, into artemisinin could possibly be optimized through harvesting and post-harvest treatment. The conversion steps from dihydroartemisinic acid into artemisinin normally occur very fast and are not necessarily enzymatic (Wallaart et al., 1999; Sy and Brown, 2002; Haynes, 2006) and thus could take place in dying or drying material. Also conditions, especially temperature, might affect this conversion.

Dead leaves in *A. annua* should be harvested because they have high artemisinin concentrations (Lommen et al., 2006). Fortunately, dead leaves are not abscised in *A. annua*. Care should be taken, however, to collect lose leaf material during harvesting and drying.

Losses of artemisinin from the leaf material can also occur, but are probably not extreme. In a study on the artemisinin content during the life cycle of leaves at different positions, a decrease in artemisinin quantity was only observed at 96 days after leaf appearance in one of the early initiated leaves (Lommen et al., 2006).

Preliminary Model for Artemisinin Production

Based on experimental results and experience thus far, we propose the following, preliminary working model to study formation of artemisinin (Fig. 4). In this model, the formation of artemisinin could be a function of:

- the number of leaves produced;
- the number of trichomes produced per leaf;
- the precursor quantities produced per trichome;
- the conversion efficiency of these precursors into artemisinin;
- the losses after formation.

This model would predict the weight of artemisinin harvested. All processes could be affected by plant genotype, environment, and management techniques.

When this model is combined with a module for leaf dry weight production and/or with the extended Monteith approach to estimate leaf yield, the model could also estimate leaf yields and artemisinin concentrations and would greatly increase understanding of crop functioning in *Artemisia* and other crops producing secondary metabolites in special structures.

Literature Cited

Bertea, C.M., Freije, J.R., van der Woude, H., Verstappen, F.W.A., Perk, L., Marquez, V., de Kraker, J.-W., Posthumus, M.A., Jansen, B.J.M., de Groot, Æ., Franssen, R. and Bouwmeester, H.J. 2005. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua* L. Planta Med. 71:40–47.

Bouwmeester, H.J., Wallaart, T.E., Janssen, M.H.A., van Loo, B., Jansen, B.J.M., Posthumus, M.A., Schmidt, C.O., de Kraker, J.-W., König, W.A. and Franssen, M.C.R. 1999. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry 52:843–854.

Dhingra, V., Rao, K.V. and Narasu, M.L. 2000. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci. 66:279–300.

Duke, S.O. and Paul, R.N. 1993. Development and fine structure of the glandular trichomes of *Artemisia annua* L. Intl. J. Plant Sci. 154:107–118.

Duke, M.V., Paul, R.N., Elsohly, H.N., Sturtz, G. and Duke, S.O. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of

Artemisia annua L. Intl. J. Plant Sci. 155:365–372.

Ferreira, J.F.S., Simon, J.E. and Janick, J. 1995. Developmental studies of *Artemisia annua*: Flowering and artemisinin production under greenhouse and field conditions. Planta Med. 61:167–170.

Ferreira, J.F.S., Simon, J.E. and Janick, J. 1997. *Artemisia annua*: botany, horticulture, pharmacology. Hort. Rev. 19:319–371.

Haynes, R.K. 2006. From artemisinin to new artemisinin antimalarials: Biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. Current Topics in Medicinal Chem. 6:509–537.

Kumar, S., Gupta, S.K., Singh, P., Bajpai, P., Gupta, M.M., Singh, D., Gupta, A.K., Ram, G., Shasany, A.K. and Sharma, S. 2004. High yields of artemisinin by multi-harvest of *Artemisia annua* crops. Ind. Crops Prod. 19:77–90.

Laughlin, J.C. 1995. The influence of distribution of antimalarial constituents in *Artemisia annua* L. on time and method of harvest. Acta Hort. 390:67–73.

Laughlin, J.C. 1993. Effect of agronomic practices on plant yield and anti-malarial constituents of *Artemisia annua* L. Acta Hort. 331:53–61.

Lommen, W.J.M., Schenk, E., Bouwmeester, H.J. and Verstappen, F.W.A. 2006. Trichome dynamics and artemisinin accumulation during development and senescence of *Artemisia annua* leaves. Planta Med. 72:336–355.

Magalhaes, P., Raharinaivo, J. and Delabays, N. 1996. Influence de la dose et du type d'azote sur la production en artémisinine d'*Artemisia annua* L. (Effects of the amount and of the type of nitrogen fertilization on the artemisinin production of *Artemisia annua* L.). Revue Suisse de Viticulture, d'Arboriculture et d'Horticulture 28:349–353.

Monteith, J.L. 1977. Climate and the efficiency of crop production in Britain. Philosophical transactions of the Royal Society of London. Ser. B. Biol. Sciences 281:277–294.

Morales, M.R., Charles, D.J. and Simon, J.E. 1993. Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Hort. 344:416–420.

Pérez-Estrada, L.B., Cano-Santana, Z. and Oyama, K. 2000. Variation in leaf trichomes of *Wigandia urens*: environmental factors and physiological consequences. Tree Physiol. 20:629–632.

Struik, P.C. and Lommen, W.J.M. 1999. Improving the field performance of micro- and minitubers. Potato Res. 42:559–568.

Sy, L.-K. and Brown, G.D. 2002. The mechanism of the spontaneous autoxidation of dihydroartemisinic acid. Tetrahedron 58:897–908.

Wallaart, T.E., van Uden, W., Lubberink, H.G.M., Woerdenbag, H.J., Pras, N. and Quax, W.J. 1999. Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. J. Nat. Prod. 62:430–433.

Wallaart, T.E., Pras, N., Beekman, A.C. and Quax, W.J. 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: Proof for the existence of chemotypes. Planta Med. 66:57–62.

Tables

Table 1. Leaf dry matter production in an *A. annua* crop during a field period of 112 days.

PAR sum during growing season	×	Fraction PAR intercepted	=	PAR intercepted by the crop	×	RUE radiation use efficiency	=	Total DM production	×	Fraction DM to leaf (green + dead)	=	Leaf DM yield (green + dead)
(MJ/m²)		(MJ/MJ)		(MJ/m²)		(g/MJ)		(g/m²)		(g/g)		(g/m²)
927		0.50±0.018		467 ±16.3		2.5 ±0.37		1134 ±183		0.24 ±0.006		284 ±44.3

Table 2. Artemisinin production in *A. annua* leaves during a field period of 112 days.

Leaf DM yield	×	Fraction artemisinin plus precursors in leaf DM	=	Artemisinin plus precursors yield	×	Fraction artemisinin in artemisinin plus precursors	=	Artemisinin yield	Artemisinin fraction in leaf DM
(g/m²)		(g/g)		(g/m²)		(g/g)		(g/m²)	(g/g)
284 ±44.3		0.0024 ±0.00089		0.70 ±0.316		0.46 ±0.039		0.31 ±0.127	0.0011 ±0.00035

Figures

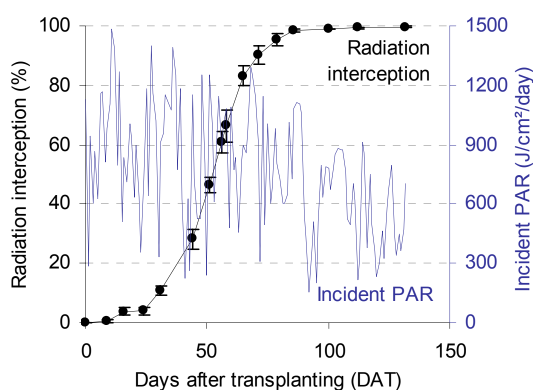

Fig. 1. Radiation interception and daily incident PAR (photo-synthetically active radiation) during field growth of an *A. annua* crop. Bar = standard deviation.

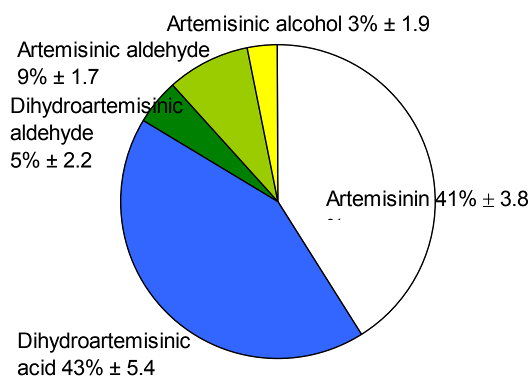

Fig. 2. Percentage of the individual components (± standard deviation) in the total molar quantity of artemisinin and assessed precursors in *A. annua* leaves.

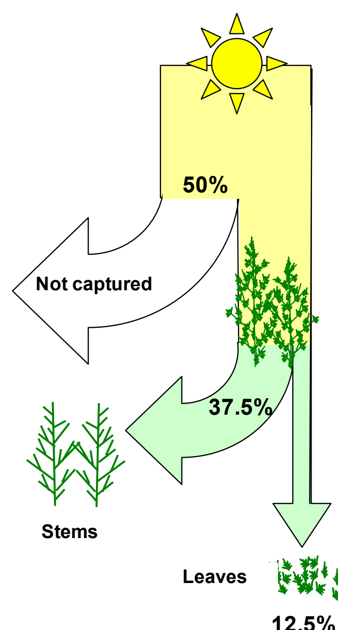

Fig. 3. Schematic overview of the losses occurring in the processes determining leaf yield in an *A. annua* crop. Of the total radiation, c. 50% was not captured by the crop to be used for dry matter production. Of the dry matter produced, 3/4 was allocated to stems, which do not contribute to artemisinin production.

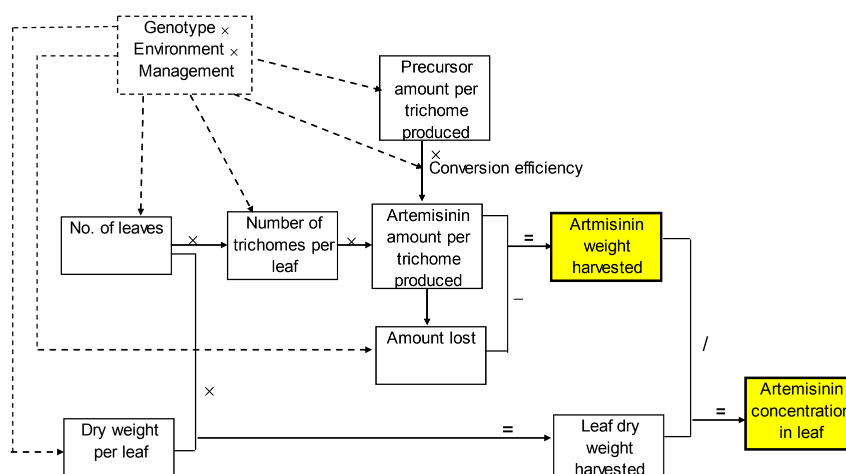

Fig. 4. Extended yield component model, explaining the production of the target compound artemisinin from the number of leaves, number of trichomes per leaf and the artemisinin production per trichome. The amount of artemisinin per trichome is affected by the production of precursors, by the degree to which the conversion of precursors to artemisinin is completed and by losses of artemisinin after formation (upper part). By combining artemisinin production with leaf dry weight production, also variation in concentrations can be explained. Dashed lines refer to effects of genotype, environment or management on variables and processes, full lines to the relations between variables. Symbols reflect the arithmetic relations between variables. This framework can be adopted for other plant species producing secondary metabolites.