1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) are among the most significant and economically important insect pests worldwide [1–5]. Their destructive impact on a wide range of agricultural crops results from a combination of causes, such as direct feeding from sap, virus transmission, and infestation of fruit and other products. The latter could hamper the market value of food exports due to quarantine restrictions in plague-free countries [6–10]. In Chile, mealybugs have been associated with up to 45% of the rejections in fruit exports during phytosanitary inspections [11].

In particular, the citrophilus mealybug *Pseudococcus calceolariae,* the species that will be employed here as a case study, colonizes a wide range of fruit crops, *e.g.* citrus, avocados, grapes, apples, and berries, among others [1,12–14]. It is present in Chile, Australia, New Zealand, the United States, South Africa, and Italy [15]. Several other countries in South and Central America, as well as in Asia, are free of this plague and are applying strict quarantine restrictions for the importation of fruits potentially infected with this species.

Several approaches to mealybug control have been developed, including biological control with natural enemies. However, the application of broad-spectrum insecticides is the standard management strategy in most crops up-to-date [16]. The multiple negative effects these chemical insecticides have on human health, environment, wildlife, and beneficial arthropods, urgently calls for new, sustainable strategies. One of such strategies is the use of pheromones, which are natural chemical compounds used for communications between individuals of the same insect species [17]. This approach is particularly attractive because its mode of action, based on interfering mating communication, is highly specific, non-toxic to other organisms, and compatible with other control strategies, such as biological control [18–20]. The use of pheromones for pest management is also compatible with organic production, a global market that is increasing fast. Unfortunately, its high cost compared to synthetic pesticides has so far precluded its widespread use. Hence, the development of novel and more efficient methods to produce pheromones could reduce their cost and boost their application for pests mitigation.

1.1 Mealybug pheromones

In some insects, such as moths, pheromones are composed of several chemical compounds, some of which can be shared between different species. Their combination and mixture ratios determine the species-specific response [21]. By contrast, in mealybugs, each species has evolved to biosynthesize and respond to a structurally unique sex pheromone compound. The first sex pheromones of mealybug species were identified in the 1980s, but it was not until the early 2000s that pheromones of many new species were reported [18]. Nowadays, twenty one mealybug sex pheromones have been identified and chemically characterized. Most of them are esters of monoterpenoid alcohols (monoterpenols) and short-chain carboxylic acids [22]. Specifically, monoterpenols have unique, irregular non-head-to-tail connections, instead of the most common head-to-tail bonds found in most monoterpenoids. Five different carbon arrangements have been described for these monoterpenols, *i.e.* acyclic branched (lavandulol), cyclopropane ring (chrysanthemol), cyclobutane ring (maconelliol and planococcyl alcohol), and cyclopentane ring (α-necrodol). The carboxylic acid part of the ester also varies across species, from acetic, propanoic or butanoic acids, to more complex esterified hydroxy- and unsaturated acids [23].

Little is known about the biosynthetic pathways of these pheromones; even more, no genomic sequence of mealybug species is available. However, based on the pheromone structures, it is expected that monoterpenol diphosphate motifs are formed by the direct condensation of two dimethylallyl diphosphate (DMAPP) molecules [22]. The resulting monoterpenols would then be specifically esterified with the corresponding acids.

The chemical structure of the citrophilus mealybug pheromone was first described by El-Sayed et al. [24] as a chrysanthemyl 2-acetoxy-3-methylbutanoate (Figure 1A). In addition to the pheromone, virgin females also secrete traces of the free alcohol (chrysanthemol) and the deacetylated pheromone (chrysanthemyl 2-hydroxy-3-methylbutanoate). These findings suggest that the pheromone is biosynthesized by the esterification of chrysanthemol with 2-hydroxy-3-methylbutanoic acid. The resulting ester would then be acetylated at the 2-hydroxy position.

The pheromone contains three chiral centers, two in the alcohol portion, and one in the acid part of the molecule. Of the eight possible stereoisomers, the female synthesizes only one, the (1R,3R)-chrysanthemyl (R)-2-acetoxy-3-methylbutanoate. Field trapping trials using different stereoisomers showed that this configuration is the only biologically active and attractive for males [25,26]. The unnatural isomers are inactive or even slightly inhibitory when used in mixtures with the active (R,R,R)-isomer. These results point to the importance of the correct stereochemistry of the pheromone for its field application in *P. calceolariae* management.

1.2 Biosynthesis of chrysanthemol, the key pheromone precursor

As mentioned above, the enzymes responsible for the biosynthesis of mealybug pheromones have not been described yet. Fortunately, some plants and insects show chemical convergence, producing common secondary metabolites [27]. For example, lavandulol and chrysanthemol, two monoterpenols from mealybug pheromones, were also found in plants; and

their biosynthetic enzymes have been characterized [28,29]. Chrysanthemol has been identified in some Asteraceae plants [30], where it is an intermediate in the biosynthesis of pyrethrins. In these plants, chrysanthemyl diphosphate (CPP) is synthesized by the condensation of two DMAPP molecules, catalyzed by the enzyme chrysanthemyl diphosphate synthase (CDS, Figure 1B). CDS also catalyzes the hydrolysis of the diphosphate, converting CPP to chrysanthemol [31]. Notably, plant CDSs are highly stereospecific, producing only (1R,3R)-chrysanthemol, the isomer of the alcohol part of the citrophilus mealybug pheromone [32]. It is worthy to mention that even though CDSs have mainly irregular, non-head-to-tail activity when DMAPP is the only substrate, in the presence of IPP, they also show head-to-tail activity. For example, when the CDS from *Artemisia tridentata* was incubated with pure DMAPP, 79% chrysanthemol, and 20% lavandulol was produced. However, when the same enzyme was incubated with equimolar concentrations of DMAPP and IPP, the resulting products were 35% chrysanthemol, 11% lavandulol, and 47% geraniol [33]. Therefore, special attention will have to be provided to the design of the biosynthetic pathway in order to minimize the presence of free IPP in the engineered biological systems, to achieve maximum chrysanthemol yields by CDS transformation of DMAPP.

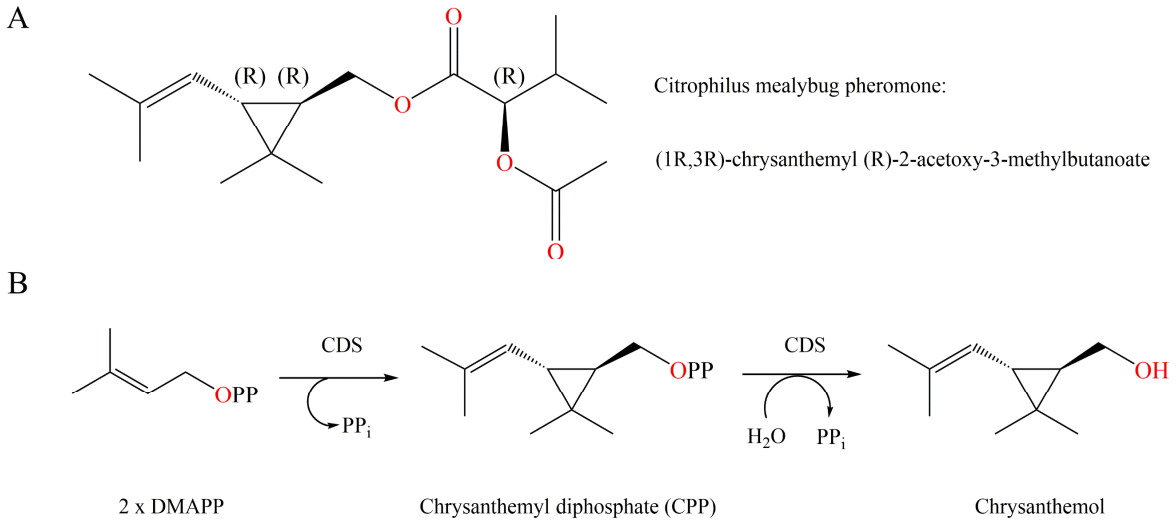

Figure 1. Citrophilus mealybug pheromone. **(A)** Structure of the pheromone. **(B)** Biosynthesis of the precursor chrysanthemol catalyzed by plant chrysanthemyl diphosphate synthase (CDS).

2. Hypothesis

Pheromone-based mate disruption offers an environmentally friendly and highly competitive way to control mealybug infestations [34–36]. However, the structural and configurational complexity of many pheromones severely hinders its chemical synthesis. For the citrophilus mealybug pheromone, the development of a stereoselective, bioproduction method would be highly attractive, as only the natural (R,R,R)-isomer has attracting activity [26]. Indeed, the pure, natural resulting stereoisomer will be eight times more active than the 8-isomer chemically produced mixtures, at the same dose. The chemical synthesis of the stereoisomeric pure pheromone requires either (1R,3R)-chrysanthemic acid or pyrethrins [25,37] as substrates. Both compounds are expensive and obtained by extraction from pyrethrum flowers. Moreover, the chemical synthesis of the pheromone involves several hazardous catalysts and solvents, in a multistep laborious process.

Here, we hypothesize that a sustainable biosynthetic process for the stereoselective production of mealybug pheromones is feasible. We will biosynthesize the active (R,R,R)-isomer of the citrophilus mealybug pheromone as a case study. Our proposal relies on the stereospecific activity of CDS enzymes to biosynthesize the key precursor (1R,3R)-chrysanthemol. First, three different approaches for the biosynthesis of chrysanthemol will be compared in terms of titers, volumetric productivities and yields: *de novo* production, biotransformation, and cell-free biocatalysis. Chrysanthemol production will be optimized by the application of computational tools, such as enzyme engineering and kinetic modeling. The resulting chrysanthemol obtained by the most performant system, will be esterified with the enantiopure acid by a lipase-catalyzed process. Finally, the capacity of the biosynthetic pheromone to attract mealybugs will be assessed in laboratory and field experiments.

It is worthy to mention that the proposed biotechnological process optimized here for citrophilus mealybug pheromone can be easily extended to other chrysanthemyl or lavandulyl esters to generate a platform for the bioproduction of pheromones from agricultural relevant mealybug species.

3. Goals

The main goal of this research is to design, build, and test the sustainable production of the citrophilus mealybug pheromone as a natural biopesticide, using engineered biological systems. Specific goals include:

1. Comparison of three strategies for the biosynthesis of the precursor (1R,3R)-chrysanthemol: *de novo* production, biotransformation, and cell-free biocatalysis.
2. Optimization of chrysanthemol biosynthesis by computational tools: enzyme engineering and pathway kinetic modeling.
3. Evaluation of enzymatic esterification of chrysanthemol for the stereoselective biosynthesis of the citrophilus mealybug pheromone.
4. Assessment of the biological activity of the bio-produced pheromone.

4. Methodology

4.1 General methodologies

Strains**.** *Saccharomyces cerevisiae* CEN.PK2-1c strain will be used as the parent strain for chrysanthemol production by *de novo* and biotransformation strategies. The transformation of yeast strains will be carried out with the LiAc/SS carrier DNA/PEG method [38]. For the cell-free approach, recombinant proteins will be produced in *Escherichia coli* BL21(DE3) and/or C41(DE3) strains. Transformation of bacterial strains will be performed using the calcium chloride method [39].

Expression and repression systems**.** Gene expression in yeast will be conducted using integrative plasmids, containing the integration sites described by [40]. Plasmid integration will be carried out through CRISPR/Cas9 method, following the protocol from Tom Ellis lab [41]. For galactose-inducible gene expression, we will use Gal1 or Gal10 promoters in a Δ*Gal1* strain. This strategy will abolish galactose metabolization and therefore enhance the inductive activity of galactose for consistent gene expression [42]. Coordinated with gene expression, the knockdown of target genes will be achieved by a galactose-inducible CRISPR/dCas9 (death-Cas9) system by the expression of dCas9 fused to a Mxi1 repressor, under Gal1 or Gal10 promoters [43,44]. The gRNAs will be designed according to the promoters of genes to be repressed. Protein production in *E. coli* will be performed by using pET expression vectors controlled by T7lac promoter and IPTG induction.

Plasmid construction**.** The assembly of yeast vectors from single DNA parts will be carried out using the Universal Loop Assembly method [45]. This Golden Gate-based system enables the versatile construction of large plasmids and constructs, using only two type IIS enzymes, two markers, and eight vectors. As the Golden Gate-based technologies work with levels to construct plasmids, level 0 plasmids (single DNA parts) will be built through the Gibson Assembly method [46]. For bacterial expression vectors, genes will be cloned into commercial pET plasmids also by Gibson Assembly.

Shake flask cultures**.** For chrysanthemol production, the strains will be cultivated in 100 mL shake flasks with 20 mL of YPDG medium (2% glucose, 0.2-1% galactose). We will also evaluate the use of a second phase of dodecane (15% v/v). Dodecane is the preferred solvent for *in situ* extraction of hydrophobic compounds from live cultures due to its low toxicity and good phase separation [47]. We have already demonstrated that dodecane efficiently extracts chrysanthemol from culture medium (see work in progress section).

Bioreactor engineering**.** Once the most performant chrysanthemol-producing strains were selected, our goal will be to optimize volumetric productivity, yield, and, titer in a fed-batch culture system. For this purpose, different control strategies will be evaluated, such as exponential fed-batch or DO-stat. In any case, optimization of a two-stage, glucose-limited exponentially fed-batch system will be prioritized. With the selected control strategy and a reasonable final biomass concentration, the induction phase will be triggered by galactose feeding. Process variables, particularly temperature, dissolved oxygen concentration, and feeding policy will be evaluated empirically to maximize chrysanthemol production.

Chrysanthemol and pheromone purification**.** Chrysanthemol obtained from liquid cultures without dodecane will be extracted using methyl tert-butyl ether or hexane [33]. Then, the solvent and other volatile compounds will be eliminated by evaporation in a rotary evaporator (chrysanthemol boiling point > 200°C [48]). From two-phase cultures, chrysanthemol will be separated from the dodecane by silica gel column chromatography [37]. Pheromone purification methods will be developed based on the residual reactants and by-products obtained in the lipase-catalyzed reactions (*e.g.* combination of organic extraction and column chromatography).

Analytical methods**.** Chrysanthemol and the pheromone will be extracted by solid phase micro extraction (SPME) with 50/30µm DVB/CAR/PDMS fiber coatings. GC-MS analyses will be conducted in a GCMS-QP2010 ultra chromatograph-mass spectrometer (Shimadzu). Helium will be used as the carrier gas with a constant flow mode. Volatiles will be separated on

a 60m DB-Wax column (Zebron) and 30m Rt-βDEXsm chiral column (Restek). The mass spectrometer will be operated in scan mode (30-200 m/z). Quantification of chrysanthemol and the pheromone will be carried out using the corresponding synthetic standards, together with 4-nonanol as internal standard. Finally, extracellular metabolites of fermentations (organic acids, glucose, ethanol, glycerol) will be determined in a Merck Hitachi HPLC with refraction index, diode array, and fluorescent detectors [49].

4.2. Comparison of three strategies for the biosynthesis of the precursor (1R,3R)-chrysanthemol: *de novo* production, biotransformation, and cell-free biocatalysis

The biosynthesis of irregular monoterpenols, like the mealybug precursor chrysanthemol, can be challenging. In order to set up the most efficient production method for chrysanthemol, we will compare the specific yields, final titers, and volumetric productivities achieved by three different approaches.

4.2A De novo production of chrysanthemol by pathway engineering in S. cerevisiae. *De novo* biosynthesis refers to the conversion of simple and abundant precursors, like sugars or amino acids, into a chemically unrelated compound through several metabolic reactions [50]. We propose to produce chrysanthemol from glucose using metabolically engineered strains of *S. cerevisiae.* This yeast has been used extensively as hosts to produce several *monoterpenes* [51,52]. For instance, the monoterpenol geraniol, which has similar chemical properties of chrysanthemol, has been successfully biosynthesized in *S. cerevisiae* with titers up to 1.68 g/L [53].

Our proposed cellular engineering strategy is summarized in Figure 2A. Since monoterpene cytotoxicity could limit production titers [54], we will develop a coordinated inducible expression/repression system. This approach enables decoupling cell growth from production stages during fermentation. Hence, the onset of chrysanthemol biosynthesis will be carried out only after enough biomass has been reached. The latter, together with the permanent extraction of the product using an aqueous-organic two-phase culture system, should alleviate potential chrysanthemol toxicity and improve volumetric productivity and titer. Both gene expression and repression will be controlled simultaneously using galactose as the only regulator, facilitating a synchronized transition from growth to production stage. Gene expression will be carried out using Gal1 or Gal10 inducible promoters. For repression, the same promoters will be applied to control the expression of dCas9 fused to a Mxi1 repressor, together with the expression of the corresponding gRNAs (see general methodologies).

The direct precursor of chrysanthemol, DMAPP, will be supplied by the endogenous MVA pathways in *S. cerevisiae*. We will increment the flux towards DMAPP by overexpressing a truncated HMG-CoA reductase (tHMG1). This strategy has already demonstrated their efficiency for improving final titers of several terpenes [51]. An efficient transformation of DMAPP into chrysanthemol will be achieved by comparing the inducible expression of CDS from several plant species. To further improve chrysanthemol production, we will consider reducing the unspecific activity of CDS and downregulate the main competing pathway. As mentioned above, CDS enzymes have shown promiscuous head-to-tail condensing activity when IPP is present, generating GPP as the main product [33]. To reduce head-to-tail activity and boost chrysanthemol production, we will increase DMAPP/IPP ratio by the inducible overexpression of IDI1 gene [55]. Finally, the inducible knockdown of the endogenous farnesyl diphosphate synthases (FDS) should improve DMAPP availability for CDS and consequently increase chrysanthemol yields.

The constructed strains will be first tested in shake-flask experiments. Then, the best strains will be scaled up to fed-batch fermentations in bench-top 1L bioreactors (see general methodologies).

4.2B Biotransformation of prenol into chrysanthemol by metabolically engineered S. cerevisiae. Whole-cell biotransformation involves using living cells as catalyzers to transform a substrate into a structurally related product by a few enzymatic reactions [50]. If the substrate is affordable, biotransformation could be an efficient alternative to *de novo* production.

Recent works have shown the successful construction of non-natural hemiterpenoid pathways, orthogonal to the native isoprene metabolism [56–58]. In this approach, supplemented isoprenol and prenol are sequentially phosphorylated by two heterologous kinases to yield IPP and DMAPP, respectively. We will implement this bypass pathway, together with CDS expression to generate a short biotransformation route from prenol to chrysanthemol (Figure 2B). The main advantage of this strategy is that the DMAPP biosynthesis can be decoupled from IPP generation, using a coordinated expression/repression system. The absence of IPP will avoid the head-to-tail promiscuous activity of CDS, improving the transformation yield of DMAPP into chrysanthemol.

We will evaluate the biotransformation of prenol into chrysanthemol in *S. cerevisiae*. Since prenol phosphorylation pathway has been constructed only in *E. coli*, we will first evaluate the tolerance of *S. cerevisiae* to this alcohol. The results will be applied to set the maximum concentration that can be used in shake-flask and bioreactor cultures, without significant cytotoxic effects. Figure 2B illustrates the proposed pathway engineering and regulation. Similar to the *de novo* production approach, we will also apply a two-stage fermentation strategy for these - biotransformation - strains. After microbial growth, we will induce the simultaneous expression and repression of selected genes, activating the capacity of the cells to

transform the exogenous prenol into chrysanthemol. Expression and repression will be carried out following the same galactose inducible strategy described in 4.2A. The expression of two alcohol kinases will convert the incorporated prenol into DMAPP, which will then be condensed and dephosphorylated by CDS to form chrysanthemol. While numerous kinases capable of phosphorylating prenol and the intermediate DMAP have been reported, several have not been directly compared. Therefore, we will compare different alcohol kinases to find the optimal combination for chrysanthemol biosynthesis. The list includes, but it is not necessarily limited to, IPK from *Arabidopsis thaliana* and *Methanothermobacter thermautotrophicus*, PhoN from *Shigella flexneri*, ThiM from *E. coli*, and CKI1 from *S. cerevisiae*. Synchronized with the expression of the chrysanthemol biosynthetic route, we will strongly repress upstream genes of the endogenous MVA pathway (ERG10 and ERG13). The latter will impede the production of IPP, which is responsible for the aforementioned promiscuous activity of CDS. To ensure that prenol-derived DMAPP cannot be interconverted to IPP, we will also repress IDI1 gene.

It is worthy to note that the cells might not sustain cell growth more than a few divisions in the productive phase, due to the downregulation of the endogenous hemiterpene pathways. However, the cells will require a maintenance carbon flux to sustain ATP and protein expression for chrysanthemol production. Hence, we will develop an induction protocol and feeding policy in high-density cell cultures that lengthen the stationary phase and maximize chrysanthemol productivity.

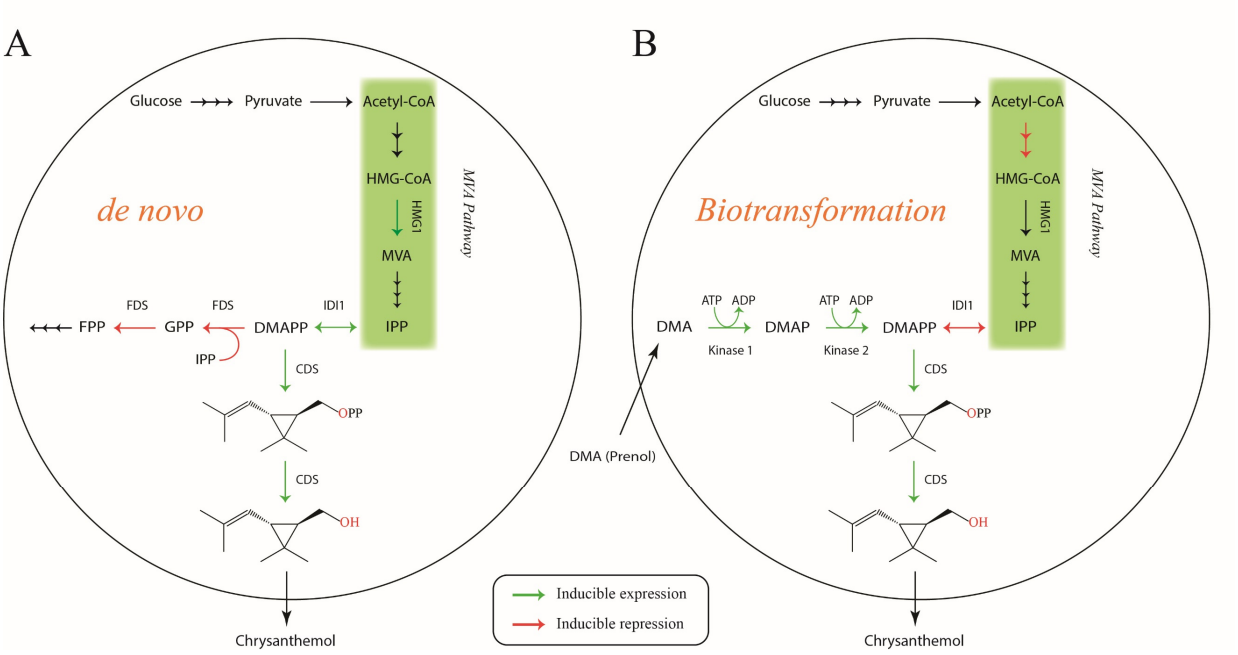

Figure 2. Two pathway engineering strategies for chrysanthemol biosynthesis in *S. cerevisiae.* **(A)** *De novo* production of chrysanthemol from glucose. The inducible expression/repression system enables the coordinated activation of the biosynthetic pathway (green arrows) and downregulates the competing pathway (red arrows). **(B)** Chrysanthemol production by prenol biotransformation. Supplemented prenol is converted to DMAPP by the sequential phosphorylation of two kinases. The biosynthetic pathway is expressed coordinately with the repression of the native MVA pathway and IDI1 gene to abolish IPP formation and maximize chrysanthemol production.

4.2C Cell-free biocatalytic transformation of prenol into chrysanthemol. Application of cell-free systems to bioproduction involves the use of purified enzymes and cofactors to convert substrates into value-added chemicals. This approach has been gaining attention in the last years due to the high titers and productivities that can be achieved, the precise control of system components, and the facility to minimize side reactions and undesirable promiscuous activities [59,60]. In some cases, the cost of enzymes and cofactors could limit the potential of cell-free systems. However, they are still useful as an optimization tool for cell-based production. Indeed, the kinetic data obtained by the *in vitro* enzymatic reactions provide the necessary parameters for the construction of pathway models, which could be extrapolated to optimize cell-based biofactories [61,62].

Here, we propose the construction and optimization of a cell-free system to produce chrysanthemol. The system will be based on the same three reactions of the whole-cell biotransformation approach. Therefore, the transformation of prenol to chrysanthemol will occur *in vitro* by the action of purified kinases and CDS (Figure 3). Similar to cell-based systems, we will evaluate combinations of kinases and CDS from different species. To obtain the enzymes, we will express the genes fused to N- and C-terminal His-tags in *E. coli*. Then, the proteins will be purified by affinity chromatography using resins

loaded with Ni^{+2} [63], and enzymatic activity will be measured. We will first perform single enzyme kinetics assays to determine the parameters of each enzyme following standard protocols [64]. These parameters will then be applied for modeling the multienzymatic system and predict optimal enzyme, substrate, and cofactor concentrations. Multienzymatic assays will be carried out following the model output, and the results will further feedback the model until achieving satisfactory productivities. Kinetic modeling procedures are described below. The data generated in the cell-free production system of chrysanthemol will also be applied to optimize biotransformation strains. For example, promoter strength could be adjusted according to the optimal ratios of enzymes obtained through *in vitro* multienzyme assays.

Despite the powerful use of cell-free biocatalysis as an optimization tool, its potential as a production method strongly relies on the cost of the associated reagents. Cofactors are often the most expensive components and can easily exceed the cost of the purified enzymes [65]. Hence, the main limitation of our proposed biocatalytic system is the requirement of the expensive cofactor ATP for the phosphorylation of prenol and DMAP. To overcome this limitation, we will evaluate coupling two different ATP regeneration biosystems to our cell-free biocatalysis workflow. Both systems are based on direct ADP phosphorylation by kinases, but with different substrates as phosphate donors. One system uses acetyl-phosphate [66], while the other is based on polyphosphate [67]. We will select the most performant ATP regeneration method to carry out chrysanthemol production at lab scale.

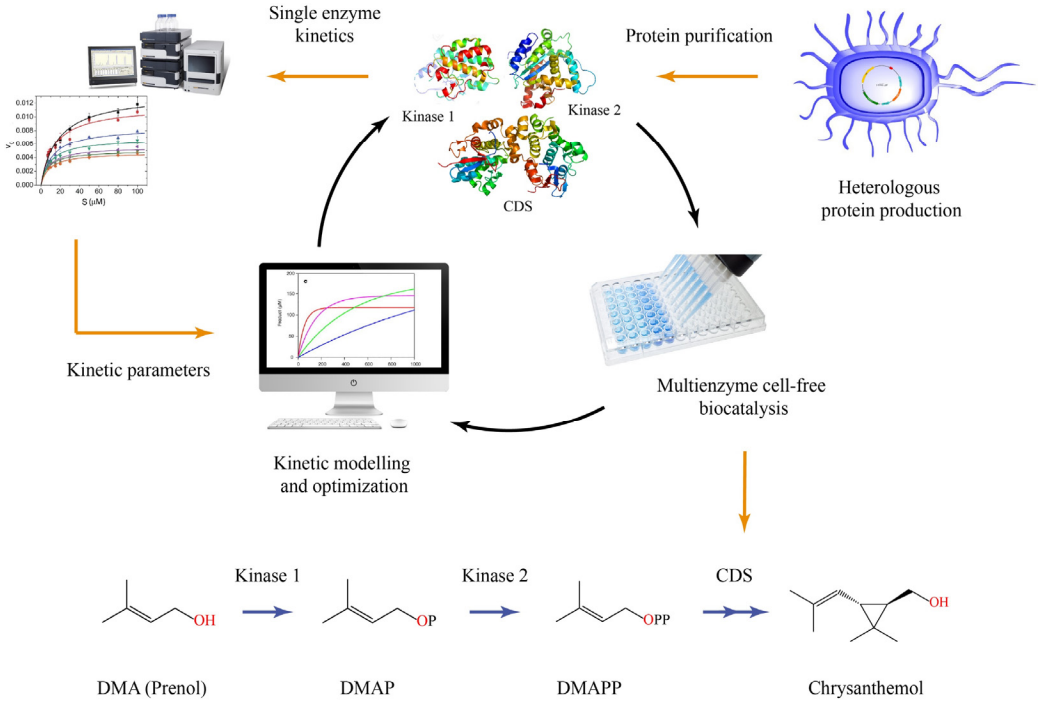

Figure 3. Design, build, and test cyclic workflow for the optimization of cell-free biocatalytic production of chrysanthemol. Parameters obtained by single enzyme kinetics are used for the modeling of the multienzymatic system and prediction of optimal enzyme, substrate, and cofactor concentrations. The data collected by multienzyme assays feed further modeling and optimization steps.

4.3 Optimization of chrysanthemol biosynthesis by computational tools

4.3A Enzyme engineering of CDS. Improving the catalytic efficiency of a given promiscuous enzyme for a particular substrate, defined as the ratio k_{cat}/K_M, can be achieved through the optimization of either the substrate binding affinity (related to K_M) or the product turnover (related to k_{cat}) [68]. The former can be tackled through bioinformatic approaches that identify specificity-determining residues (SDR), only conserved in subfamilies of enzymes with similar activities, and molecular docking simulations that assess substrate binding energies.

Both CDS and the homologous farnesyl diphosphate synthases (FDS) have one allylic binding site comprising up to three divalent metals, which preferentially accepts DMAPP as substrate; and a second binding site for the binding of either IPP or DMAPP, depending on substrate specificity/promiscuity [33,69].To determine SDR responsible for IPP and DMAPP binding in the second binding site of these enzymes, we will first retrieve amino acid sequences of CDS and FDS from the Uniprot database [70], using the sequence of the promiscuous CDS from *Tanacetum cinerariifolium* (acc. code P0C565) and *A. tridentata* (Q7XYS8) and the IPP-preferent human (P14324) and *E. coli* FDS (P22939) as search queries in PSI-BLAST [71]. A multiple sequence alignment (MSA) will be derived with MAFFT [72] and a phylogenetic analysis to determine the

different clades of these enzyme families will be performed via phylogenetic analysis using MEGA [73] as shown in a previous work on FDS from *Aedes aegypti* [74]. Both the whole MSA and the sequence groups from each clade in the phylogenetic trees will be subjected to real-value evolutionary trace analysis on the UET server [75] for determination of SDR behind the preference for IPP and DMAPP. This information will be used in molecular docking of IPP and DMAPP on the modeled structure of *A. tridentata* [33] using Rosetta [76] as in our previous works [77]. The SDR changes identified between the different clades will be used for combinatorial *in silico* mutagenesis and molecular docking to quantify those that energetically favor DMAPP binding over IPP. The latter could reduce the promiscuous head-to-tail activity of CDS in favor of the desired non-head-to-tail activity.

To experimentally screen the effects of these mutations, we will perform both, site-directed mutagenesis and iterative saturation mutagenesis [78] on the positions that harbor the SDR in *A. tridentata*. The *in vitro* activity of wild-type CDS and its different mutants will be spectrophotometrically assayed through a coupled reaction, in which the release of PPi at the expense of DMAPP or IPP is used to produce 2-amino-6-mercapto-7-methyl-purine that can be measured at 360 nm, as recently described [79]. Kinetic experiments will be performed under increasing concentrations of DMAPP, IPP or both, to determine the change in catalytic efficiencies of the different mutants.

4.3B Pathway kinetic modeling. The chrysanthemol cell-free production system will be optimized using kinetic models describing the dynamic behavior of the biochemical reactions. To this task, single-enzyme *in vitro* assays will be performed to quantitatively characterize the kinetics of the corresponding enzymes, *i.e.* kinase 1, kinase 2, CDS, and ATP-regenerating enzymes. It is well recognized that fitting mechanistic kinetic models to experimental data is challenging, due to their high parameter number, and highly non-linear nature. Despite these challenges, recent Monte Carlo-based strategies have shown that satisfactory predictions can be achieved even when many parameters are poorly resolved in these models [80–84]. In fact, a comprehensive survey has revealed that satisfactory predictions do not necessarily require precise parameters [85].

The General Reaction and Assembly Platform (GRASP) will be applied to construct detailed and thermodynamically plausible kinetic models of the constituent enzymes of the metabolic pathway [86]. Grounded on Bayesian principles, this framework enables a statistically sound assessment of parameter uncertainty and model predictions [82]. Briefly, by evaluating the discrepancy between model simulations generated from GRASP and the *in vitro* assay data, an approximation to the parameter posterior distribution – represented by a population of parameters that fit the data within some tolerance level – can be attained and employed for predicting the kinetics of each enzyme [82,87]. Then, assembly of the models for all the enzymes will enable *in silico* simulation and optimization of the proposed cell-free system. Using these models, we will determine the optimal relative enzyme, substrate, and cofactor concentrations required for maximizing the productivity of the system, while maintaining a robust performance [88]. Finally, these results will also be employed to optimize the performance of the engineered strains (objective 1).

4.4 Evaluation of enzymatic esterification of chrysanthemol for the stereoselective biosynthesis of the citrophilus mealybug pheromone.

The transformation of chrysanthemol into the citrophilus mealybug pheromone requires two sequential esterification reactions. Biocatalytic esterification processes have gained acceptance in industrial applications due to the development of inexpensive, efficient, and stable lipases. In native physiological environment, lipases catalyze the hydrolysis of triglycerides at lipid/water interface. However, at low water activities (i.e., organic solvents), lipases catalyze reverse hydrolytic reactions such as esterification and transesterification [89]. We propose a straightforward, lipase-catalyzed esterification process of the chrysanthemol precursor to biosynthesize the citrophilus mealybug pheromone.

Both esterification reactions will be carried out by a one-pot/two-step biocatalytic process (Figure 4). First, a lipase will be employed to acetylate (R)-2-hydroxy-3-methylbutanoic acid into the intermediate (R)-2-acetoxy-3-methylbutanoic acid (Figure 4A). As most lipases cannot utilize efficiently acetic acid as an acyl donor due to enzymatic inhibition or inactivation [90], we will evaluate the acetylation of the acidic substrate, using either ethyl or vinyl acetate as acyl donors. Transesterification with acetyl esters has indeed shown good performance for the production of acetate esters [91,92]. The resulting intermediate will then be esterified with chrysanthemol in the same reactor, without any intermediate purification step, to yield the target pheromone (Figure 4B). Transesterification and esterification reactions will be both pursued in a solvent-free system, using immobilized lipases. This strategy has already been successfully applied to the biosynthesis of other monoterpene esters, such as geranyl acetate [93] and geranyl propionate [94]. Solvent-free conditions could further simplify the separation of the product from the reaction mix, while immobilization will enable recycling the biocatalyst several times. The latter might allow to count with an economically-feasible and sustainable process. One possible limitation of this system, however, is the enzymatic inactivation that could occur by the elevated acid concentrations. The latter is dependent on the employed acid, reaction conditions, and the lipase [95]. If we measure significant inactivation rates in the solvent-free conditions, we will re-evaluate the use of solvents in order to ensure the recycling of the catalyst for at least three reaction cycles, with retention of over 50% of lipase activity.

Three commercial immobilized lipases will be compared: Novozym 435, Lipozyme TL IM, and Novozym 40086. These lipases have been efficiently employed in a wide range of esterification and transesterification reactions [96,97]. Optimal conditions for each reaction will be found by surface response methodology, using a central composite design [98]. We will analyze the effect of four process variables on conversion yields: temperature, substrate molar ratio, enzyme concentration, and time.

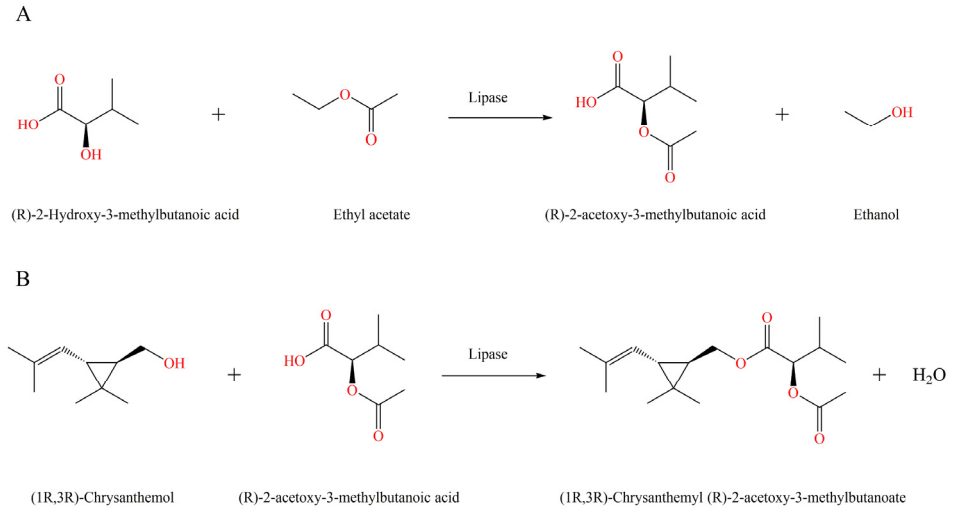

Figure 4. One-pot/two-step biocatalytic process for the esterification of chrysanthemol into the citrophilus mealybug pheromone. **(A)** (R)-2-Hydroxy-3-methylbutanoic acid is acetylated by transesterification with ethyl or vinyl acetate esters. **(B)** The resulting (R)-2-acetoxy-3-methylbutanoic acid is esterified with chrysanthemol.

4.5 Assessment of the biological activity of the bio-produced pheromone.

We will assess the biological activity of the bio-produced pheromone by carrying out laboratory and field experiments. For laboratory experiments, we will maintain a *P. calceolariae* colony to obtain insects for all the experiments. Initial laboratory experiments will allow to check for biological activity of the bio-produced pheromone. For this purpose, 10 sexuality mature males (2-4 days old) will be released into a Petri dish arena, containing four pieces of filter paper: 2 pieces of 2 cm^2 moistened with distilled water, one piece of 1 cm^2 with pheromone solutions, and one piece of 1 cm^2 treated with a solvent (hexane). Males will be observed for 20 min, and the percentage of males on the filter paper with the pheromone will be counted. We will run 6 experimental treatments: 1, 3 and 5 ng of bio-produced pheromone and 1, 3 and 5 ng of the standard chemically produced pheromone. Each treatment will have at least 5 replicates. Percentage of males responding will be analyzed using a generalized linear model (GLM), with type of pheromone and dose as factors.

Once the biological activity and pheromone doses at which males respond would be determined, we will carry out a laboratory experiment using a Y-type olfactometer. In this case, single males will be tested to two volatiles, each coming from one of the arms of the olfactometer. The experimental combinations to evaluate will consider: (1) bio-produced pheromone against blank (solvent); (2) standard chemically produced pheromone against blank (solvent); (3) bio-produced pheromone against chemically produced pheromone, at the same concentration; and (4) bio-produced pheromone against chemically produced pheromone, at an equivalent concentration of the bioactive isomer. A male response to one of the arms will be considered positive when it moves 1 cm into the arm within 5 min observation period [99]. At least 20 males per combination will be tested, and then discarded. Preference for one of the arms in each combination will be tested by Chi^2 (goodness of fit).

Finally, we will test the activity of pheromones in the field. For this purpose, we will prepare lures using the bio-produced and the standard chemically produced pheromone, both dissolved in hexane. Then, white rubber septa will be loaded with the respective solutions to have doses of 40 µg of standard chemically produced pheromone (approximately 5 µg of the active isomer), 40 µg of the bio-produced pheromone, and 5 µg of the bio-produced pheromone. Septa will be put singly inside Delta traps with removable floors. Then, 10 traps for each of the lures (30 total) will be randomly deployed, at least 30 m apart, in an organic apple orchard infested with *P. calceolariae* [26]. After 1 week, floors will be extracted and transported to the laboratory to count males under a compound microscope. New floors will be put in the traps, and their position in the field will be rotated and left for another week. Males catches for each type of lure will be compared by ANOVA, with dates as blocks.

Work plan

	Year 1		Year 2		Year 3	
Goal	1-6	7-12	1-6	7-12	1-6	7-12
1. Comparison of three strategies for the biosynthesis of the precursor (1R,3R)-chrysanthemol: *de novo* production, biotransformation, and cell-free biocatalysis						
De novo production of chrysanthemol by pathway engineering in *S. cerevisiae*						
Biotransformation of prenol into chrysanthemol by metabolically engineered *S. cerevisiae*						
Cell-free biocatalytic transformation of prenol into chrysanthemol						
2. Optimization of chrysanthemol biosynthesis by computational tools						
Enzyme engineering of CDS						
Pathway kinetic modeling						
Experimental evaluation of the most promising computational designs						
3. Evaluation of enzymatic esterification of chrysanthemol for the stereoselective biosynthesis of the citrophilus mealybug pheromone						
Preliminary reactions tests: selection of acyl donor, solvent-free condition vs. accessory solvents						
Search of optimal reaction conditions for three lipases by surface response methodology						
Fine-tuning of the production process and purification of the pheromone.						
4. Assessment of the biological activity of the bio-produced pheromone						
Obtention and maintaining of mealybug colonies						
Laboratory bioassays: Petri dishes and olfactometer						
Field trials						

5. Team experience and capacity

Synthetic biology and metabolic engineering: The construction of strains for chrysanthemol production (goal 1) will be carried out by Dr. Agosin's team. Dr. Agosin has proficiency and broad experience in the application of molecular and synthetic biology tools to metabolically engineered microorganisms. In the last years, he has focused on the production of carotenoids and flavors (apocarotenoids) in engineered yeast [47,100–102].

Computational tools: Protein modeling and enzyme engineering of CDS (goal 2) will be performed by Dr. Ramírez-Sarmiento's team. Dr. Ramírez has expertise in protein biophysics, bioinformatics, and biochemistry [77,103,104]. Kinetic modeling of chrysanthemol production will be in charge of Dr. Pedro Saa, who has substantial experience in the optimization of metabolic systems and bioprocesses [82,105,106] with a supporting role of Eduardo Agosin.

Bioprocess: Bioreactor fermentation processes and enzymatic catalysis (goals 1 and 3) will be developed by Agosin's group, with a supporting role of Dr. Saa. In the past years, both researchers have been working together in this area [101,107].

Chemical analysis: The detection and quantification of different analytes will be carried out by the center for flavours and aromas team, which is directed by Dr. Agosin. This team has the experience and all the necessary instrumentation to perform GC and HPLC analyses.

Biological assays: Pheromone biological activity will be tested in laboratory and field experiments (goal 4) by Dr Tania Zavieso's team. Dr. Zaviezo is an entomologist expert in integrated pest management and has been working for several years in the development of identification, monitoring, and management strategies for mealybugs in Chile [25,26,108].

6. Work in progress

6.1 Gene synthesis, expression vectors, and golden gate library

Several genes needed for execution of this proposal were already synthesized and cloned into yeast expression vectors. Examples of these genes are CDS from *A. tridentata*, CDS from *T. cinerariifolium*, IPK from *M. thermautotrophicus,* and PhoN from *S. flexneri*. We have also partially constructed a Golden Gate uLooP library [45] by cloning several DNA parts into entry vectors (level 0), including promoters (constitutive and inducible), terminators, genomic integration sites, and genes.

6.2 Construction of chrysanthemol yeast strains using constitutive promoters

As a proof of concept to produce chrysanthemol, we have constructed a series of *S. cerevisiae* strains that constitutively expressed the required biosynthetic genes. We integrated up to two copies of CDS in both full-length and truncated (without plastid transit peptide) variants. To increase DMAPP flux, we co-expressed the CDSs with IDI and/or tHMG1 genes. Unfortunately, due to COVID-19 pandemic and the subsequent closure of the university from mid-March until now, we have not been able to analyze these strains. At least, we set up earlier the analysis of chrysanthemol from the culture media, as illustrated in Figure 5.

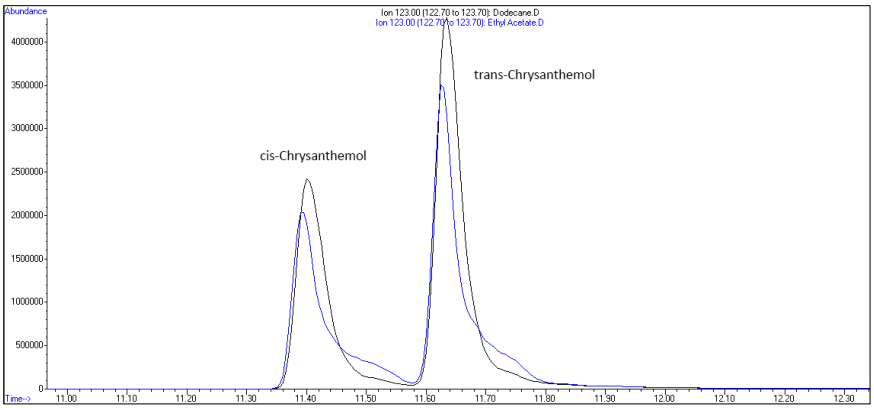

Figure 5. Chrysanthemol standard (0.5 mg/L) extracted from culture media using ethyl acetate and dodecane and analyzed by GC-MS. The standard was a mixture of 38% cis-chrysanthemol (1R,3S and 1S,3R) and 62% trans-chrysanthemol (1R,3R and 1S,3S), containing the four possible stereoisomers. Dodecane showed better extraction efficiency (90%) than ethyl acetate (76%).

6.3 Fermentation processing

Continuous, batch, and fed-batch culture systems are routinely run in our four in-house built, 1L bioreactors in the fermentation lab. Dissolved oxygen saturation levels can be tightly controlled by a triple split-range control action, including agitation, airflow, and pure oxygen flow. Up to 100 g/L of *S. cerevisiae* biomass have been obtained in fed-batch cultures in these bioreactors [101].