

Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica*

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Abstract

The European corn borer (ECB) *Ostrinia nubilalis* is a widespread pest of cereals. Mating disruption with the sex pheromone is a potentially attractive method for managing this pest. The goal of this study was to develop a biotechnological method for the production of ECB sex pheromone. Our approach was to engineer the oleaginous yeast *Yarrowia lipolytica* to produce (Z)-11-tetradecenol (Z11-14:OH), which can be chemically acetylated to (Z)-11-tetradecenyl acetate (Z11-14:OAc), the main pheromone component of the Z-race of *O. nubilalis*. Fatty acyl-CoA desaturases (FAD) and fatty acyl-CoA reductases (FAR) from nine different species of Lepidoptera were screened individually and in combinations. A titer of 29.2±1.6 mg/L Z11-14:OH was reached in small-scale cultivation with an optimal combination of a FAD (Lbo_PPTQ) from *Lobesia botrana* and FAR (HarFAR) from *Helicoverpa armigera*. When the second copies of FAD and FAR genes were introduced, the titer improved 2.1-fold. The native FAS1 gene's overexpression led to a further 1.5-fold titer increase. When the same engineered strain was cultivated in controlled 1 L bioreactors in fed-batch mode, 188.1±13.4 mg/L of Z11-14:OH was obtained. Fatty alcohols were chemically acetylated to obtain Z11-14:OAc. Electroantennogram experiments showed that males of the Z-race of *O. nubilalis* were responsive to biologically-derived pheromone blend. Behavioral bioassays in a wind tunnel revealed attraction of male *O. nubilalis* at a level similar to that of the chemically synthesized pheromone used as a control, although full precopulatory behavior was observed less often. The study paves the way for the production of ECB pheromone by fermentation.

1 Introduction

Crop damage caused by insects is a severe problem in agriculture. Moths (Lepidoptera) are major insect pests [1-3]. The European corn borer (ECB) *Ostrinia nubilalis* is the main pest of maize *Zea mays* in Europe [4]. It is estimated that in the absence of treatment, up to 20% of the crop may be lost due to the damage by *O. nubilalis* larvae [5]. Like other moth species, females of *O. nubilalis* produce and release a fatty acid-derived sex pheromone, which attracts conspecific males for mating [6]. ECB is polymorphic with respect to its pheromone communication system and two pheromone races are recognized: the Z-race insects use a 97:3 blend of (Z)-11-tetradecenyl acetate (Z 11-14:OAc) to (E)-11-tetradecenyl acetate (E 11-14:OAc), while E-race insects use a 1:99 blend of the same components [6,7]. On maize in Europe, the Z-race is most prevalent [8].

Mating disruption has been proven an effective and environmentally friendly solution for crop protection

against moths. For mating disruption, a more or less species-specific pheromone blend is applied to the fields or orchards to disrupt mating partner detection and, in this way, decrease the propagation of the pest species [9,10]. Currently, pheromones for pest management are produced synthetically from petrol-derived chemicals [11-13]. Chemical synthesis typically comprises multiple steps, uses toxic chemicals and solvents, and expensive catalysts. Biotechnological production of several insect pheromone components has already been established in plants and yeasts [14-18]. Oleaginous yeast *Yarrowia lipolytica* is particularly suitable for production of lepidopteran pheromones due to its naturally high level of fatty acid biosynthesis. Previously, we produced the precursor of the major pheromone component of *Helicoverpa armigera* (Z 11-16:OH) at 2.5 g/L in *Y. lipolytica* and proved the effectiveness of yeast-derived insect pheromone in field trapping experiments [16].

In this study, we aimed to produce Z 11-14:OAc, the major pheromone component of the ECB Z-race, using engineered *Y. lipolytica* as a host and demonstrate the activity of yeast-derived pheromone on *O. nubilalis* males.

2 Materials and methods

2.1 Plasmid construction

Plasmids were constructed according to Holkenbrink et al., 2018 [19]. Integration and guide RNA (gRNA) vectors were used to introduce gene expression constructs into characterized genome sites of *Y. lipolytica* [19].

Primers, synthetic DNA, BioBricks, and plasmids used in this study are listed in Supplementary Tables S1, S2, S3, and S4, respectively. BioBricks were amplified by PCR using Phusion U polymerase (Thermo Fisher Scientific) with the following thermal program: 98°C for 5 min, 30 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 30 s/kb), and 72°C for 4 min. After DNA electrophoresis on 1% agarose gel, BioBricks were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The integration vectors were digested with FastDigest SfaAI (Thermo Fisher Scientific) and nicked with Nb.BsmI (New England BioLabs). BioBricks with compatible overhangs and nicked integration vectors were assembled and transformed into *Escherichia coli* strain DH5α via Uracil-Specific Excision Reaction (USER®) cloning. Clones containing the correct assembly were verified by PCR and Sanger sequencing.

2.2 Strain construction

All strains constructed in this study are derived from the *Y. lipolytica* strain ST6629, described in Holkenbrink et al. [16]. This strain has modifications related to decreased degradation of fatty acids/alcohols and an increased pool of fatty acyl-CoAs. *Y. lipolytica* strain ST4840, which was obtained from Agricultural Research Service (NRRL, USA), served as a source for genomic DNA (gDNA). gDNA was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The entire list of the strains is provided in Table S5.

Yeast transformations were performed as described in Holkenbrink et al. [19] with the modifications described below. *Y. lipolytica* strains were streaked on Yeast Peptone Dextrose (YPD) plates (20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 15 g/L agar) and grown for 24 h at 28degC. A small patch of biomass was taken with an inoculation loop and re-streaked on YPD plates containing 0.7 g/L complete supplement mixture (Formedium). After 24 h incubation at 28degC, the cells were scraped off, resuspended in 1 mL 0.5 M sterile sucrose solution, and centrifuged for 5 min at 3,000 g at room temperature. The supernatant was discarded, the cells resuspended in 0.5 M sucrose solution, and a volume corresponding to OD₆₀₀ 2.6 was transferred to a sterile tube for transformation. Tubes were centrifuged for 5 min at 3,000 g at room temperature, and the supernatant was removed. 500-1,000 ng of gRNA plasmid and integration plasmid, which previously was linearized with SmaI (Thermo Fisher Scientific) and gel-purified prior transformation, were added to the pellet. The mixture was resuspended in 100 µL of transformation mix (Supplementary Table S6) and incubated at 39°C for 1 h. After the heat shock, the tubes were centrifuged, the supernatant removed, and the pellet was resuspended in 500 µL liquid YPD medium. The cells were incubated for 2 h at 28°C 300 rpm shaking. The pellet was again collected by centrifugation, resuspended in 100 µL of 0.5 M sterile sucrose solution, and plated on YPD plates containing Hygromycin B (200 mg/L) (Carl Roth)

or Nourseothricin (250 mg/L) (Jena Bioscience) for selection. After 2-3 days of incubation at 28°C, single colonies appeared, which were tested for correct integration by PCR using vector-specific primers and primers complementary to genomic loci close to the integration site (Supplementary Table S1).

2.3 Small scale cultivations

Y. lipolytica strains were inoculated from a YPD agar plate to an initial OD₆₀₀ of 0.2 into 2.5 mL YPG medium (10 g/L yeast extract, 10 g/L peptone, 40 g/L glycerol) in 24 well-plate (EnzyScreen). The plate was incubated at 28°C and 300 rpm for 22 hours. The plate was centrifuged at 3,500 g for 5 min at 20°C, the medium was removed and the cells were resuspended in 1.25 mL production medium (50 g/L glycerol, 5 g/L yeast extract, 4 g/L KH₂PO₄, 1.5 g/L MgSO₄, 0.2 g/L NaCl, 0.265 g/L CaCl₂·2H₂O, 2 mL/L trace elements solution: 4.5 g/L CaCl₂·2H₂O, 4.5 g/L ZnSO₄·7H₂O, 3 g/L FeSO₄·7H₂O, 1 g/L H₃BO₃, 1 g/L MnCl₂·4H₂O, 0.4 g/L Na₂MoO₄·2H₂O, 0.3 g/L CoCl₂·6H₂O, 0.1 g/L CuSO₄·5H₂O, 0.1 g/L KI, 15 g/L EDTA). The plate was incubated at 28°C and 300 rpm for 28 h. Each strain was cultivated in triplicate.

2.4 Bioreactor fed-batch cultivations

Bioreactor fed-batch mode cultivations were carried out in biological triplicates on initial YPG medium (50 g/L glycerol, 10g/L yeast extract (Carl Roth), 10 g/L peptone) in controlled stirred bioreactor vessels with 1.0 L total aerated end working volume (Infors Minifors 2 systems, Infors AG, Switzerland) at 28°C. All reactors were equipped with pH and optical dissolved oxygen probes (Hamilton AG, Switzerland) and off-gas analyzers (BlueSens GmbH, Germany). pH was controlled at 4.5±0.1 with automated addition of a 4 M solution of NaOH. The dissolved oxygen control was set at a minimum threshold of 20% with a cascade control by gradually increasing the stirrer speed of two six-blade Rushton turbines and the aeration rate using Eve fermentation control software (Infors AG, Switzerland). The initial aeration rate was set to 1 L/min, with stirring at 400 rpm. The CO₂ (%) and O₂(%) in the off-gas were monitored continuously during the fed-batch cultivations. Bioreactor cultures of strain *Y. lipolytica* ST9253 were inoculated from shake flask precultures in the exponential growth phase (250 mL baffled shake flasks, 40 mL cultivation volume, YPG 40 g/L glycerol, 10 g/L yeast extract, 10 g/L peptone) to a starter fermentation volume of 620 mL. After the depletion of the initially supplied glycerol carbon source, the cultures were fed with a nutrient-rich feed solution (700 g/L glycerol, 10 g/L yeast extract, 10 g/L (NH₄)₂SO₄). The composition of the feed solution was set to facilitate the production of the target compounds after an initial biomass buildup phase. Off-line samples were taken regularly to analyze residual glycerol concentration (Megazyme Inc. assay kit), optical density at 600 nm (Genesys photometer, Thermo Fischer Scientific), cell dry weight, and fatty alcohol concentrations.

2.5 Extraction and derivatization of lipids

1 mL of cultivation broth was sampled into 4 mL glass vials and centrifuged at 3,500 g for 5 min at 20°C. The supernatant was removed, and 1 mL of 1 M HCl in anhydrous methanol was added to the pellet. Samples were vortexed for 20 s and incubated at 70°C for 2 h for methanolysis reaction to proceed. Samples were cooled down to room temperature, 1 mL of 1 M NaOH in methanol, 500 µL of saturated NaCl solution in water, 1 mL of hexane, and 10 µL of internal standard (2 g/L of methyl nonadecanoate (19:Me) in hexane) were added sequentially. Vials were vortexed for 10 s, centrifuged at 3,500 g for 5 min at 20°C, and the upper organic layer was taken for analysis.

2.6 Extraction of fatty alcohols from small-scale samples

In small-scale screening experiments, 1 mL of cell culture was sampled into 4 mL glass vials and centrifuged at 3,500 g, 5 min, 21°C. The supernatant was removed, and 1 mL of ethyl acetate:ethanol mixture (85:15, v/v) was added to the pellet together with 10 µL of internal standard (2 g/L of methyl nonadecanoate (19:Me) in ethyl acetate). Vials were vortexed for 20 s and incubated for 1 h, followed by 5 min vortexing. 300 µL of water was added, samples vortexed for 10 s, centrifuged at 3,500 g, 5 min, 21°C, and the upper organic layer was taken for analysis.

Samples from bioreactors were processed as follows. 100 μ L of cell culture was taken, and the total broth was processed in the same way as for small-scale samples except that the first centrifugation step was omitted.

2.7 Extraction and work-up of fatty alcohols from bioreactor cultivation for acetylation reaction

The 2.4 L of broth from the bioreactor cultivation was centrifuged at 3,000 g for 5 min at room temperature, and the supernatant was separated from the pellet. Fatty alcohols were extracted from the supernatant by adding 2.2 L of ethyl acetate and incubating on a multi-vortexer for 1 h. Organic phase was decanted. To extract fatty alcohols from the pellet, 100 mL of ethyl acetate was added to the pellet, and the mix was shaken on a multi-vortexer for 6 h. The organic phase was decanted, 100 mL of fresh solvent was added to the biomass pellet, and the mixing repeated for 1 h. The organic phase was decanted. All ethyl acetate fractions from the supernatant and pellet extraction were combined and dried with anhydrous sodium sulfate. The solvent was removed in a rotary evaporator.

The extracted crude fatty alcohol mixture was passed through a silica gel column, which was prepared as described in [20]. The silica was washed with hexane and then subsequently with a gradient of hexane/ethyl acetate at the proportion of 95:5 to 40:60 (% v/v). Purest fractions based on thin-layer chromatography were collected, and the solvent was evaporated in a rotary evaporator.

2.8 Acetylation of fatty alcohols

The purified fatty alcohol mixture was acetylated by using a 1.2 molar equivalent of acetic anhydride and 0.1 equivalent of sodium acetate anhydrous (equivalents were based on 14:OH). The reaction mixture was allowed to stir for 1 h at 80°C in a water bath and then transferred to a separatory funnel where it was diluted with ethyl acetate and washed with a saturated solution of sodium bicarbonate. The organic phase was dried with anhydrous sodium sulfate, and the solvent evaporated in rotary evaporator.

2.9 Gas chromatography-mass spectrometry (GS-MS) analysis

Analysis was carried out on an Agilent GC 7820A coupled to a MS 5977B, equipped with a split/spitless injector and a DB-Fatwax UI column (30 m x 250 μ m x 0.25 μ m). The operation parameters were: 1 μ L injection, split ratio 20:1 for FAMES and alcohol samples and 10:1 for acetates, injector temperature 220 °C, constant flow 1 mL/min of helium, oven ramp 80°C for 1 min, 20 °C/min to 150°C, then 1°C/min to 200°C, then 20°C/min to 230°C. MS was scanning between m/z 30 and 350. For compound identification, reference standards were purchased from Pherobank, and retention times together with mass spectra were compared to the sample of interest. Quantification was made based on 6 points calibration curves. Visual inspection of chromatograms was performed using MassHunter Qualitative Analysis Navigator B.08.00 (Agilent), while quantification was performed using MS Quantitative Analysis (Quant-My-Way) (Agilent).

2.10 Electrophysiological responses of male *Ostrinia nubilalis*

Antennal responses of male ECB adults to biologically-derived pheromone blend (BioPhe) were evaluated by electroantennography (EAG) using a commercially available electroantennographic system (Ockenfels Syntech GmbH, Buchenbach, Germany). The antenna of a virgin two-to-three-day old male adult was excised from the head using micro-scissors and attached between two stainless-steel wire electrodes with the assistance of electrically conductive gel (G008, Fiab, Italy). The base of the antenna was connected to the indifferent electrode, whilst the distal end was connected to the recording electrode after cutting off a few segments of the tip. The signal was amplified 10X by a Universal AC/DC pre-amplifier probe connected to the recording electrode, and the analog signal was amplified and detected with a data acquisition controller (IDAC-4, Ockenfels Syntech GmbH).

Test stimulus was the biologically-derived pheromone blend BioPhe containing 7.2% *Z* 11-14:OAc, according to GC-MS analysis. Commercially available *Z* 11-14:OAc, the major sex pheromone of the *O. nubilalis* *Z*-race, was used as a reference compound. The BioPhe blend, as well as commercially available pheromone compounds, were diluted in pentane. For each stimulus, a 10 μ L aliquot of the solution was pipetted to a piece of filter paper (7 x 30 mm, Whatman no. 1), and the solvent was allowed to evaporate before being

inserted into a Pasteur pipette for odor delivery. Control stimulus consisted of a clean Pasteur pipette with a filter paper impregnated with solvent (pentane). Stimuli were provided as 0.3 s air puffs into a continuous flow of filtered and humidified air. The airflow, at 25 cm³/s rate, tube diameter 1 cm, was generated by an air stimulus controller (CS-55, Syntech, The Netherlands).

The reference stimulus, consisting of a filter paper carrying 100 ng of *Z* 11-14:OAc, was provided at regular intervals during each recording session. The EAG response to each reference stimulus was defined as 100%, and all responses to test stimuli between adjacent references were normalized in % relative to the references. At least 1 min for weak and moderate stimuli and 5 min for strong stimuli were allowed between successive stimulations to let the antenna recover. The BioPhe blend was tested at a 100 ng dose (containing ~7 ng *Z* 11-14:OAc), as well as at a 1 µg dose (*i.e.*, 70 ng *Z* 11-14:OAc), in order to reach an amount of major sex pheromone comparable to the 100 ng dose of the *Z* 11-14:OAc stimulus.

The minor sex pheromone, *E* 11-14:OAc, was also tested, as well as both isomers at a 97:3 *Z*:*E* ratio, based on the ratio released from the female moth gland as reported in the literature for ECB *Z*-race [7,21]. In addition, considering the high amount of tetradecyl acetate (14:OAc) in the BioPhe blend (~50%), the EAG response to 50 ng of this compound was also evaluated (for consistency reasons 50 ng is approx. the amount of 14:OAc contained in the 100 ng BioPhe test dose). All aforementioned stimuli were tested on a total of 20 antennal preparations.

2.11 Behavioral bioassays

Behavioral studies were performed in a dark room under red light (2.5-3 lux) at 21±2°C and 70±5% relative humidity. Male moths were tested in a wind tunnel as described in [22]. For odor delivery, a piece of triangular-shaped filter paper (2.5 cm base, 4 cm height) was pipetted with appropriate volume of the test solution and, following solvent evaporation, was hung from the suction hook in the tunnel.

Pheromone standards and BioPhe samples were dissolved in pentane. The major and minor sex pheromone components *Z* 11-14:OAc and *E* 11-14:OAc, respectively, were mixed at a 97:3 ratio and were used as a positive control at a 50 µg dose, which, through preliminary testing, was concluded to be the minimum amount eliciting positive courtship behavior, *i.e.*, source-oriented flight pattern, abdominal hair pencils display and abdomen curling upon contact. Additionally, to evaluate potential antagonistic effects of BioPhe blend contaminants on the males' responses, 14:OAc (most prominent component ~50% of BioPhe blend) and *Z* 9-11:OAc (~9% of BioPhe blend) have been tested.

A dose of 700µg BioPhe was used in the bioassays (containing 50µg *Z* 11-14:OAc and 350µg 14:OAc) and a dose of 350µg was added to 50µg pheromone standard (97:3) to emulate abundance and ratio in the BioPhe. Also a dose of 50µg pheromone standard (97:3) with the addition of 4.5µg of *Z* 9-16:OAc (9% present in the BioPhe blend) was tested, as to our knowledge the role of *Z* 9-16:OAc has not been investigated as antagonist to male response. Other components of BioPhe, such as *Z* 9-14:OAc have already been reported in the literature as antagonist to male response [23,24]. Behavioral tests were conducted between the 3rd and 5th hour in the scotophase [24,25]. Two hours before testing, 2-5 days old male moths were transferred in individual 400 mL clear plastic cups covered with perforated lids and left in the conditions of the dark room to acclimate. For each test, the odor source was attached to the tunnel ceiling, and after 30 seconds, an individual male was released in the tunnel, and its behavior was recorded for 10 min.

The following sequences of behavior were recorded: close approach (less than 10 cm) and contact with the source (landing). In addition, the approach and landing steps were further analyzed into four grades, and each grade was assigned its corresponding value. For the landing step, grades were discriminated as follows: grade 1) brief contact with the source (no landing), grade 2) landing on source for 1-2 sec, grade 3) landing on source for more than 2 seconds, and grade 4) landing on source accompanied by hairpencils display and/or abdomen curling (copulation attempt). Similarly, for the approach step were discriminated as: grade 1) close approach to source for 1-2 sec, grade 2) close approach with zig-zag patterned flight for > 2 sec, grade 3) close approach with zig-zag patterned flight and hairpencils display for 1-2 sec, grade 4) close approach with zig-zag patterned flight and hairpencils display for > 2 sec.

Between treatments, the flight tunnel inner walls and suction hook were wiped with acetone and left to aerate for 10 min before the next treatment. For each treatment, 30 males were tested. Males were used once and were discarded after testing.

2.12 Statistical analyses

The electrophysiological and behavioral data were subjected to analysis of variance (ANOVA) (SAS Institute, 2000). The means of electrophysiological data and the response grades were separated using the Tukey (honestly significant difference, HSD) test at $P = 0.05$.

3 Results

3.1 Screening of fatty acyl-CoA desaturases

In *O. nubilalis*, a Δ^11 desaturase acts on myristoyl-CoA (14:CoA), which in turn is generated by β -oxidation of the abundant fatty acid synthesis product hexadecanoyl-CoA (16:CoA), and generates a mixture of *E*/*Z* 11-14:CoA [6,26]. In *Y. lipolytica*, myristic acid is a minor component of the total fatty acid pool, comprising only about 0.25% of the total fatty acids [27]. To increase the supply of 14:CoA in *Y. lipolytica*, we substituted isoleucine 1220 of the fatty acid synthase ketoacyl synthase domain to phenylalanine (Fas2p^{I1220F}). This mutation was proposed to hinder the binding of longer acyl-CoAs in the active site tunnel of the FAS complex and was reported to result in the production of shorter chain acyl-CoAs [27]. The mutation has already been proven to be effective in the production of *Z* 9-14:OH, where it increased the titer 15-fold [16]. In this study, we introduced the FAS mutation into *Y. lipolytica* strain ST6629, which was previously developed for decreased degradation of fatty alcohols and decreased storage lipid synthesis [16]. The resulting ST7982 showed 8.4-fold increase in myristic acid production compared to the parental strain (Supplementary Figure S1).

Next, seven Δ^11 desaturases with previously documented *E*/*Z* 11-14:CoA activity were tested in strain ST7982 (Figure 1, Figure S2). These were the desaturase Lbo_PPTQ from the grapevine moth *Lobesia botrana* [28], Onu11 from the European corn borer *O. nubilalis* [26], EpoE11 from the light brown apple moth *Epiphyas postvittana* [29], CroZ11 from the oblique banded leafroller moth *Choristoneura rosaceana* [30], CpaE11 from the spotted fireworm moth *Choristoneura parallela* [31], Hzead11 from the corn earworm *H. zea* [32], and Msxead11 from the tobacco hornworm *Manduca sexta* [33]. The resulting strains were cultivated, their lipids methanolized into FAMES, and analyzed on GC-MS. Lbo_PPTQ expression resulted in the highest content of *Z* 11-14:acid. The *Z*/*E* isomer ratio was 9:2. For the strain expressing the OnuE11 desaturase, no new compounds could be detected, indicating that this desaturase is most likely not well expressed or active in *Y. lipolytica*. Likewise, no activity was detected for the desaturases, EpoE11 and CpaE11, which were previously reported to produce *E* 11-14:acid in another yeast, *Saccharomyces cerevisiae*, albeit in minuscule amounts [29,31]. The strain expressing CroZ11 produced a mixture of *E*/*Z* 11-14:acid with an excess of the *Z* isomer; however, the amounts of both isomers were lower compared to the strain expressing Lbo_PPTQ. The desaturase Hzead11 appeared to be a promiscuous Δ^11 desaturase with a high preference towards 16:acid (Figure 1). Apart from *Z* 11-16:acid, we also detected *E*/*Z* 11-14:acid and some other unsaturated fatty acids, which remain to be identified. (Figure S2). The Msxead11 desaturase-expressing strain produced *E*/*Z* 11-14:acid in ratio 1:1, where the amount of the *E* isomer was the highest among the tested desaturases.

In summary, Lbo_PPTQ resulted in the highest content and purity of *Z* 11-14:acid and was therefore chosen for establishing the biosynthetic pathway towards ECB pheromone in *Y. lipolytica*.

3.2 Screening of fatty acyl-CoA reductases

We further screened four fatty acyl-CoA reductases (FARs) to identify the reductase with the highest activity and selectivity towards *Z* 11-14:acid. The four reductases were: HarFAR from cotton bollworm *H. armigera* [34], SlitpgFARII from African cotton leafworm *Spodoptera littoralis* [35], and two reductases from *O. nubilalis*, OnuFAR_E and OnuFAR_Z [36]. First, we expressed the four reductases individually in the strain ST7982. The resulting strains were cultivated as follows: first, the cells were grown for biomass propagation for 22

h, followed by a production phase in media supplemented with equal amounts of *E* and *Z* 11-14:Me (500 mg/L of each isomer) lasting for 28 hours. Fatty alcohols were extracted from the broth and quantified on GC-MS. HarFAR and SlitpgFARII expressing strains produced around 40 and 14 mg/L of *E* 11-14:OH and *Z* 11-14:OH from the corresponding methyl esters, respectively, and did not exhibit a preference towards any isomer. OnuFAR_E produced very low amounts (<1 mg/L) of *E* / *Z* 11-14:OH, which were below the quantification limit. However, manual inspection of the chromatograms showed that this reductase had a bias towards *E* isomer, as expected. No fatty alcohols were detected in the strain expressing OnuFAR_Z (Figure 2, Figure S3).

Next, for *de novo* production of *Z* 11-14:OH, we expressed the four reductases in the strain ST9992, which contained Fas2p^{I1220F} mutation and expressed desaturase Lbo_PPTQ. Interestingly, while HarFAR resulted in 2.8-fold higher product titer than SlitpgFARII in the feeding assay above, in this experiment with *de novo* production, a higher *Z* 11-14:OH titer was obtained in strain with SlitpgFARII, 37.7±2.6 mg/L versus 29.2±1.6 mg/L for HarFAR strain.

However, the strain expressing SlitpgFARII additionally produced 402.5±17.3 mg/L of 14:OH and 54.7± 4.0 mg/L of *Z* 9-16:OH, which is 2.2 and 1.8-fold higher compared to HarFAR, respectively (Figure 2B). Very high activity towards tetradecanoate of SlitpgFARII was also observed by Antony et al. [35], who reported that among a wide variety of saturated, mono-, and di-unsaturated fatty acyl substrates, tetradecanoate was converted into alcohol most efficiently.

We chose HarFAR for further strain engineering due to its high activity and better selectivity towards *Z* 11-14:CoA than SlitpgFARII.

3.3. Metabolic engineering for improved production of *Z*11-14:OH

After selecting Lbo_PPTQ and HarFAR as the biosynthetic enzymes for *Z* 11-14:OH production, we proceeded with further metabolic engineering strategies to increase the product titer (Figure 3A). First, we introduced two gene copies of HarFAR into the high myristate producing strain ST7982 to enable biosynthesis of fatty alcohols. The resulting strain ST8225 produced 318.3±52.4 mg/L total fatty alcohols, among which 14:OH constituted 67.5% and was the most abundant. In order to produce *Z* 11-14:OH, we expressed Lbo_PPTQ in ST8225 leading to ST8373, which yielded 2 1.5±2.2 mg/L of the target compound. Compared to the parental strain, this strain made 46.7% more of the total fatty alcohols. Then we introduced a second copy of Lbo_PPTQ and this led to a further 3-fold improvement of *Z* 11-14:OH titer.

We anticipated that further improvement could be achieved by overexpressing native *FAS1* from *Y. lipolytica*. Previous studies in *S. cerevisiae* showed that the activity of FAS complex is enhanced when the copy number of *FAS1* gene is increased [37]. Indeed, overexpression of *FAS1* (strain ST9253) resulted in 60.7% improvement in the total fatty alcohol titer and increased production of *Z* 11-14:OH by 54.7%, resulting in 93.9±11.7 mg/L of *Z* 11-14:OH in small-scale cultivation.

3.4 Production of *Z*11-14:OAc by fed-batch fermentation and alcohol acetylation

The engineered strain ST9253 (MATa *ku70* [?] *Cas9* *hfd4* [?]*hfd1* [?] *pex10* [?] *fao1* [?] *hfd2* [?] *hfd3* [?] GPAT_100bpPr Fas2p^{I1220F} 2xLbo_PPTQ2xHarFAR —*FAS1*) was cultivated in controlled bioreactors in fed-batch mode. The initial batch phase served as a biomass propagation phase. After the depletion of the initial carbon source supplied in the batch phase, the composition of the fed-batch feed solution was set to facilitate the production of fatty alcohols. In the first 42 h of fermentation, the accumulation of biomass was targeted, and stationary phase was reached after 49 h (Figure3B). The start of the fed-batch facilitated a shift towards nitrogen-limited conditions and thereby transition from the biomass build-up phase to production of fatty alcohols.

In the fatty alcohol production phase of the fermentation process, we observed a constant increase in specific yield. The specific production yield of *Z* 11-14:OH peaked at the very end of fermentation and reached 0.007 g product/g DW. At this time point, the titer of *Z* 11-14:OH was 188.1±13.4 mg/L, while the cell dry weight reached 28.2±0.7 g/L (Figure 3B). The titer of 14:OH reached 1,350.1±188.1 mg/L. It was the

most abundant fatty alcohol in the fermentation broth. Tight control of parameters such as pH, dissolved oxygen and glycerol levels in the bioreactor cultivations led to a 2-fold improvement in the target compound titer compared to the small-scale cultivations.

After 120 h of fed-batch fermentation, fatty alcohols were extracted with organic solvent and purified on a silica column. The resulting fatty alcohol mixture containing approximately 250 mg of *Z* 11-14:OH was acetylated with acetic acid anhydride. It resulted in full conversion, where 320.1±13.4 mg of *Z* 11-14:OAc was obtained, and no traces of alcohols were left at the end of the reaction (Figure 4). The resulting product is referred to as BioPhe, for biologically-derived pheromone.

3.5 Electrophysiological responses of male *Ostrinia nubilalis*

We studied electrophysiological responses of male *O. nubilalis* insect antennae to pure chemical pheromone components, BioPhe product, and several blends. As expected, EAG responses of ECB *Z*-race male moths were higher for 100 ng *Z* 11-14:OAc than for *E* 11-14:OAc (Figure 5A). Also, the 97:3 (*Z* 11-14:OAc/*E* 11-14:OAc) mixture at 100 ng dose elicited the same magnitude of response as the major pheromone component (*Z* 11-14:OAc). The biologically-derived pheromone BioPhe at 100 ng elicited a significantly lower response, as expected, because *Z* 11-14:OAc is present just at 7.2% *ergo* and only about 7 ng of *Z* 11-14:OAc were directed to the antenna. When the quantity of BioPhe was increased ten-fold, the EAG response was also increased. In this treatment, the amount of *Z* 11-14:OAc present in the biologically-derived mixture was of the same order of magnitude as the reference (*i.e.* , ~70 ng). However, it should be noted, that some of the other BioPhe components may also contribute to the augmented response recorded. In this context, we tested the effect of 14:OAc, given the fact that it comprises about 50% of BioPhe. At 50 ng dose, analogous to that contained in 100 ng BioPhe, it was clearly detected by the antenna but to a much lesser degree than the reference compound.

3.6 Behavioral bioassays

Male ECB moths responded with approach and landing to 50 µg of the pheromone blend corresponding to the *Z* -race (Figure 5B). As BioPhe contains just 7.2% *Z* 11-14:OAc, a 700 µg stimulus of BioPhe was tested in lab bioassays (containing approx. 50 µg *Z* 11-14:OAc). Significantly fewer source-oriented approaches and fewer landings were observed compared to the response elicited by the optimal *Z*-race pheromone, indicating the presence of compound(s) interfering with the insects' anticipated behavior.

In the BioPhe sample 14:OAc comprises almost 50% of the total amount. To emulate a BioPhe sample, 350 µg 14:OAc were added to the 50 µg pheromone blend (97:3). In this case, males scored significantly lower in both approach and landing. This is an indication that 14:OAc at this dose interferes with the precopulatory behavior of *O. nubilalis*.

Similarly, when 4.5 µg of *Z* 9-16:OAc was added to the 50 µg pheromone blend (97:3), significantly fewer landings were recorded, but the frequency of approaches did not differ significantly from what was observed in response to the native pheromone .

4 Discussion

We have evaluated the performance of seven FADs and four FARs, which are involved in insect sex pheromone biosynthesis, to establish the production of *Z* 11-14:OH in *Y. lipolytica* . Among FADs, Lbo.PPTQ showed the highest production of (*Z*)-11-tetradecenoate and was selected for further strain engineering. Several desaturases, namely, Onu11, EpoE11, and CpaE11, were not active. The desaturase Onu11 was previously assayed in other recombinant hosts, such as *S. cerevisiae* and *Nicotiana benthamiana* with divergent results. In *S. cerevisiae* , it generated *E* /*Z* 11-14:acid in ratio 4:5 and also produced *Z* 11-16:acid [26], while no activity of this enzyme was observed in *N. benthamiana* [14]. The other two desaturases that were non-functional in our study, EpoE11 and CpaE11, were reported to act on 14:acid and produce *E* 11-14:acid in *S. cerevisiae* , but the amounts were very low [29,31]. One possible explanation is that the non-functional desaturases were poorly expressed or misfolded. A recent study by Buček et al. showed that different moth

desaturases could have different expression levels [38]. Codon-optimization could also have been suboptimal [39].

After examining FADs, we screened several FARs for increased specificity towards fatty acids with C14 chain length. Previously, HarFAR was shown to prefer (*Z*)-9-tetradecenoate over (*Z*)-11-hexadecenoate and (*Z*)-9-hexadecenoate [34]. We hypothesized that this reductase should also be able to act on (*E/Z*)-11-tetradecenoate, which is structurally similar to (*Z*)-9-tetradecenoate. Indeed, a feeding experiment and co-expression with Lbo_PPTQ confirmed that HarFAR could produce *E/Z* 11-14:OH from corresponding acids. (*E/Z*)-11-tetradecenoate was also converted into alcohol by *Y. lipolytica* expressing SlitpgFARII. This FAR previously produced the highest amounts of *Z* 11-14:OH among the four tested reductases from *Spodoptera* spp. [35]. OnuFAR_E and OnuFAR_Z were shown to be selective for E and Z isomers of 11-tetradecenoate, respectively [36], however, in our study, OnuFAR_E showed little and OnuFAR_Z no activity. The same results were obtained when these enzymes were screened in the plant *N. benthamiana* [14]. One possible explanation for the lack of functionality of these enzymes in yeast and plant expression hosts could be incorrect folding of the reductases in the ER membrane, which has a different lipid composition in yeasts and plants than in insects [40-43].

In order to improve the production of *Z* 11-14:OH, we employed metabolic engineering strategies, such as integration of multiple copies of genes and enhancement of precursor supply by overexpression of *FAS1* subunit of *Y. lipolytica*. In small-scale cultivations of the engineered yeast strain, we achieved 93.9 ± 11.7 mg/L of *Z* 11-14:OH. On the way of building the production strain (ST9253), we observed that introduction of desaturase Lbo_PPTQ not only contributed to the biosynthesis of *Z* 11-14:OH, but it also improved the total fatty alcohol titer. It was previously shown that overexpression of the native *OLE1* desaturase increased fatty acid biosynthesis in general, likely because the fatty acid synthase complex FAS is less inhibited by unsaturated fatty acyl-CoAs than by saturated fatty acyl-CoAs [44-46].

For collection of pheromone for EAG and behavioral tests, the engineered strain was fermented in controlled 1 L bioreactors in fed-batch mode. We used glycerol as the carbon source with a high carbon-to-nitrogen ratio to favor fatty alcohol production [47-52]. We obtained 188.1 ± 13.4 mg/L of *Z* 11-14:OH. The strain also produced large amounts (over 1.3 g/L) of the saturated by-product 14:OH, indicating a significant limitation of the desaturation step. This may be improved by further strain engineering and fermentation optimization in the future. To our knowledge, this is the first study showing the production of *Z* 11-14:OH in a microbial host. Previously this pheromone precursor was recombinantly synthesized in plant *N. benthamiana* yielding 14 µg from 1 g leaf tissue [14]. While *Y. lipolytica* has been engineered for the production of fatty alcohols in multiple other studies, the common products are naturally unsaturated or saturated fatty alcohols [48,49,53-56].

To convert the fermented alcohol into acetate, which is the active sex pheromone component of *O. nubilalis*, a chemical acetylation step was performed, which resulted in full conversion and yielded 320 ± 13.4 mg of *Z* 11-14:OAc. Interestingly, based on the current knowledge about insect pheromone biosynthesis, this reaction should be catalyzed by acyltransferases in insects [6,57]. However, until now, no enzymes from moths catalyzing this reaction have been found even though some gene candidates have been proposed and tested [58].

The yeast-derived pheromone blend caused a response of *O. nubilalis* males in electroantennogram experiments similar to what could be expected based on the responses to synthetic pheromone compounds. Furthermore, the blend was attractive to insects in behavioral bioassays in a wind tunnel to the same level as the chemically synthesized pheromone blend. However, full precopulatory behavior was observed less often. In order to induce a complete the ECB precopulatory behavior, a higher purity may be required, as certain by-products present in the blend may hinder the complete expression of the precopulatory behavior. Notably, reduced precopulatory behavior is not necessarily a hindrance and may even be a benefit for mating disruption. Therefore, activity studies in the field are warranted.

In summary, we have successfully employed yeast *Y. lipolytica* for production of *O. nubilalis* sex pheromone

precursorZ 11-14:OH and showed that the resulting yeast-derived pheromone was biologically active in modulating the behavior of *O. nubilalis* males.

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Conflict of interest

IB, CH, CL, BJD are co-inventors on patent applications WO2016207339, WO2018109167, and WO2018109163. KP, KRK, HJ, NM, AM, AG, CS, BL, CH, IB have financial interest in BioPhero ApS. DR has financial interest in Novagrica SA.

Data Availability Statement

Not applicable

Author Contributions

The concept of the study was developed by KP, IB, MK, DR, and CL. KP, IB, MK, DR, and CL acquired the funding for the research. The investigation was carried out by KP, EK, PCB, KRK, HJ, NM, AM, AG, CS, BL, and CH. B-J D and CL provided Lbo.PPTQ sequence. IB, MK, DR, and CL supervised the work. KP, CH, IB, CL, and MK drafted the manuscript. All authors participated in reviewing and editing the manuscript.

5 References

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Figures and legends

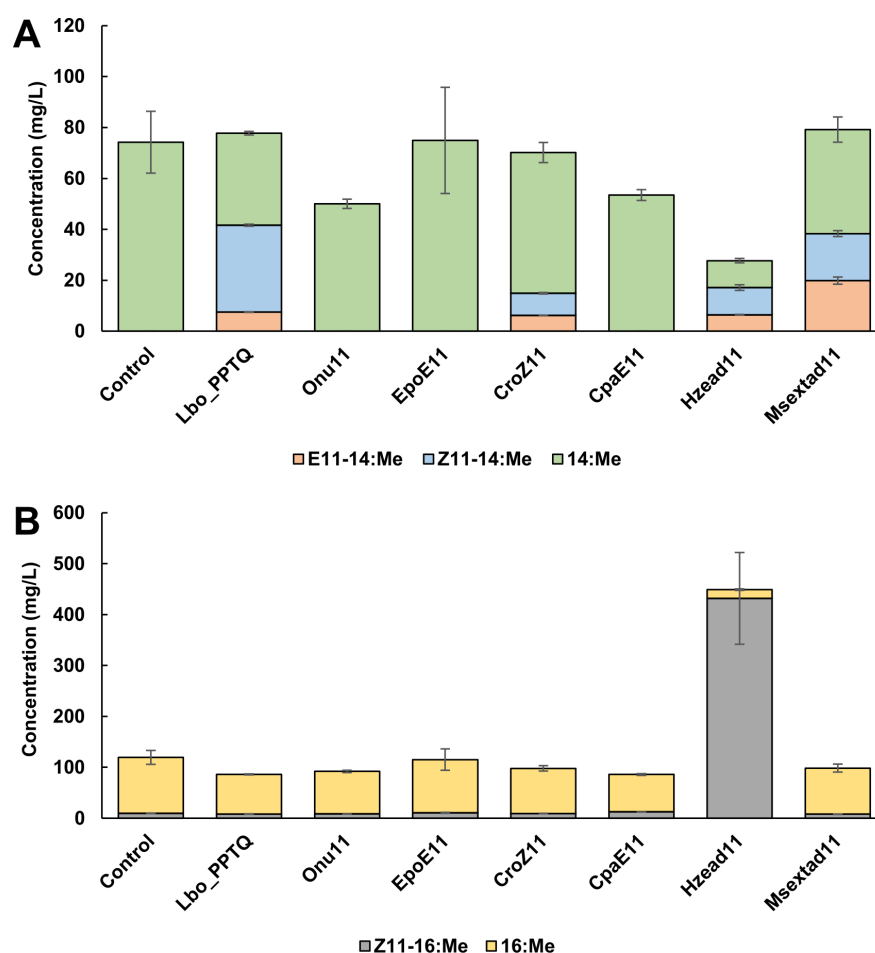


Figure 1. Characterization of [?]11 desaturases. **A.** Production of *E* / *Z* 11-14:acid and **B.** *Z* 11-16:acid by *Y. lipolytica* containing different moth fatty acyl-CoA desaturases. Quantification of fatty acids was done after derivatization into methyl esters. Results are obtained from three biological replicates. Error bars indicate standard deviations.

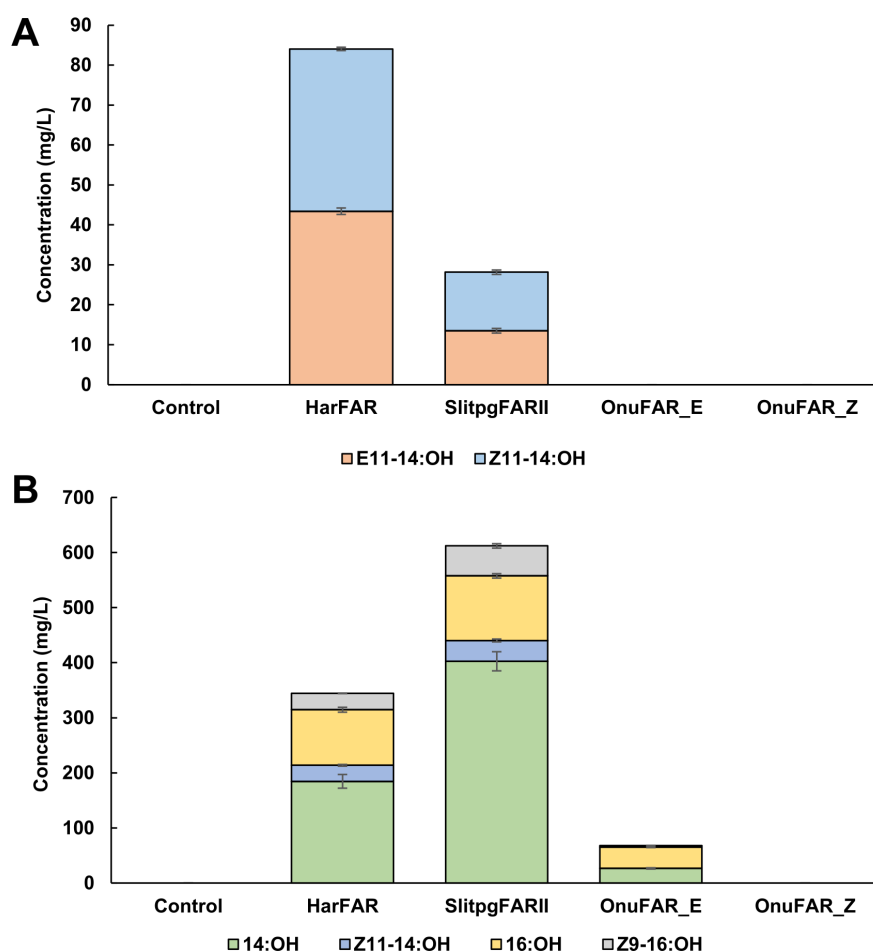


Figure 2. Characterization of reductases. **A.** Profiles of *E* / *Z* 11-14:OH produced by *Y. lipolytica* containing different reductases when cultivation media was supplemented with a mixture containing equal amounts (500 mg/L) of *E* / *Z* 11-14:Me. **B.** Fatty alcohol profiles of *Y. lipolytica* strains expressing Lbo_PPTQ together with different reductases. The control strain has no reductase introduced. Results are obtained from three biological replicates. Error bars indicate standard deviations.

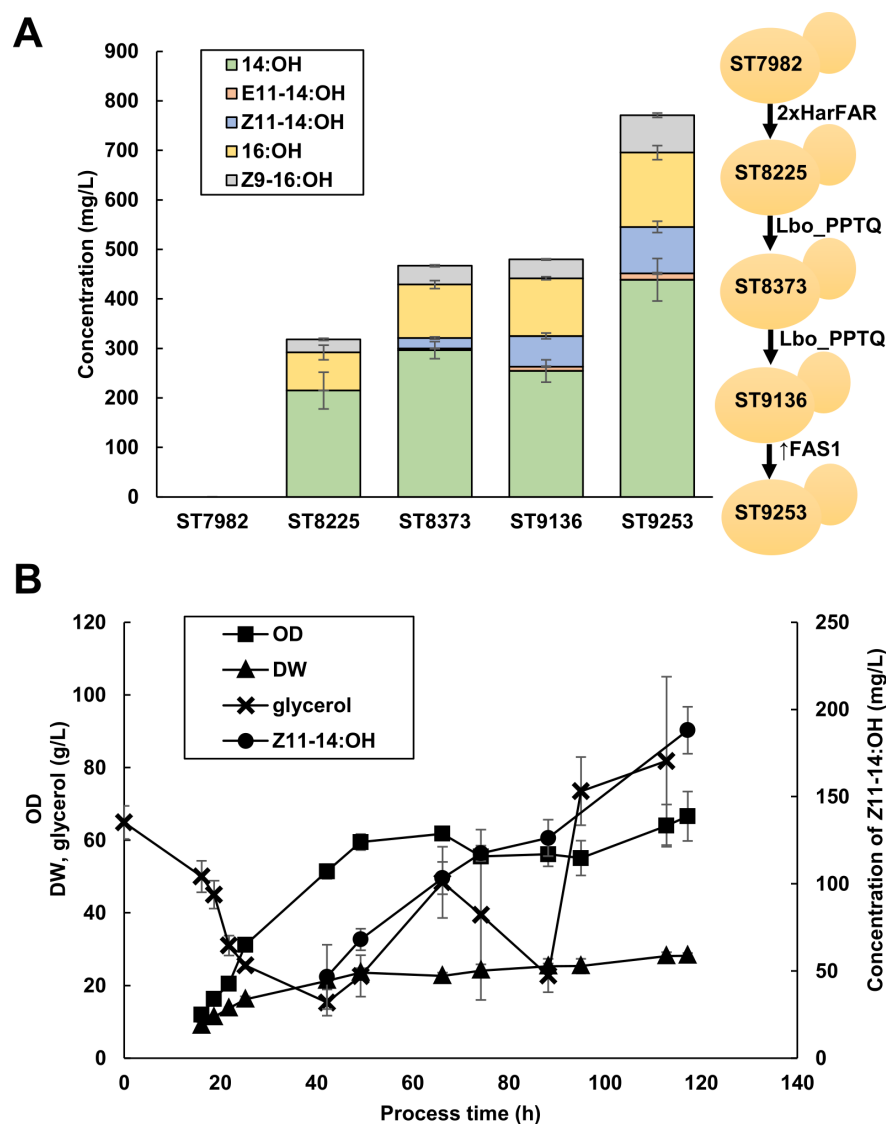


Figure 3. Production of Z 11-14:OH in small scale cultivations and in 1 L fed-batch fermentation. **A.** Metabolic engineering of *Y. lipolytica* for improved production of Z 11-14:OH. High myristate producing strain ST7982, containing mutation in α -subunit of the fatty acyl synthase complex (Fas2p^{I1220F}) was used as background strain. Results are obtained from three biological replicates. **B.** Fed-batch fermentation results of ST9253. Data obtained for optical density (OD), cell dry weight (DW), glycerol, and Z 11-14:OH concentration are represented as averages from three bioreactors and standard deviations are calculated which are shown as error bars.

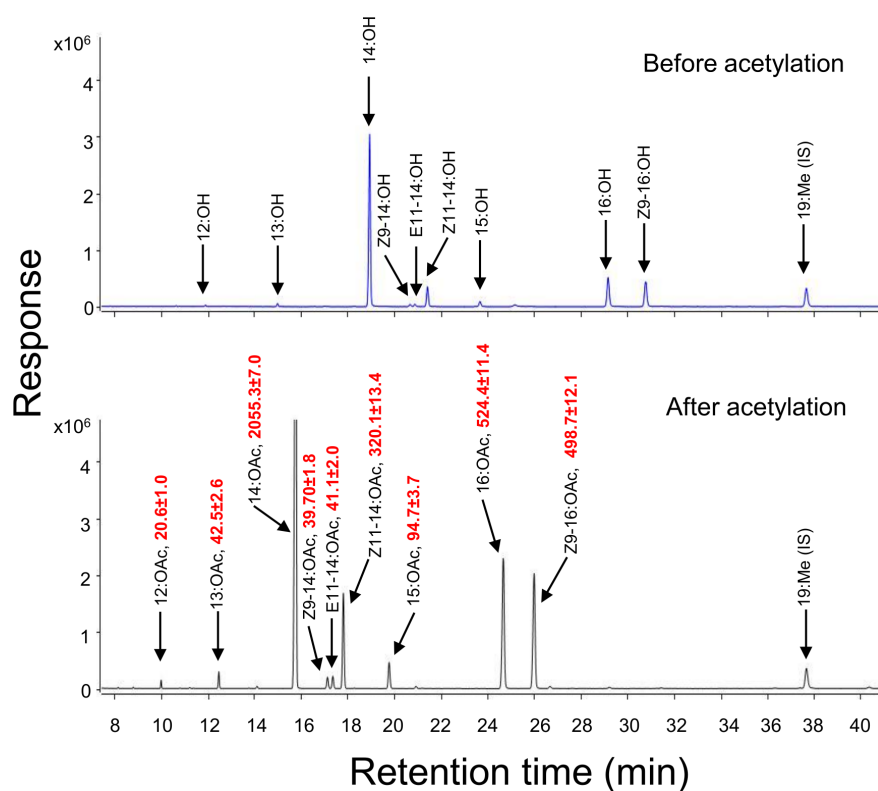


Figure 4. GC-MS chromatogram representing fatty alcohol sample before (above) and after (below) acetylation. Numbers in red represent the amount of each fatty acetate (mg \pm SD) in final sample. Measurements were performed in technical triplicates. Error bars indicate standard deviations.

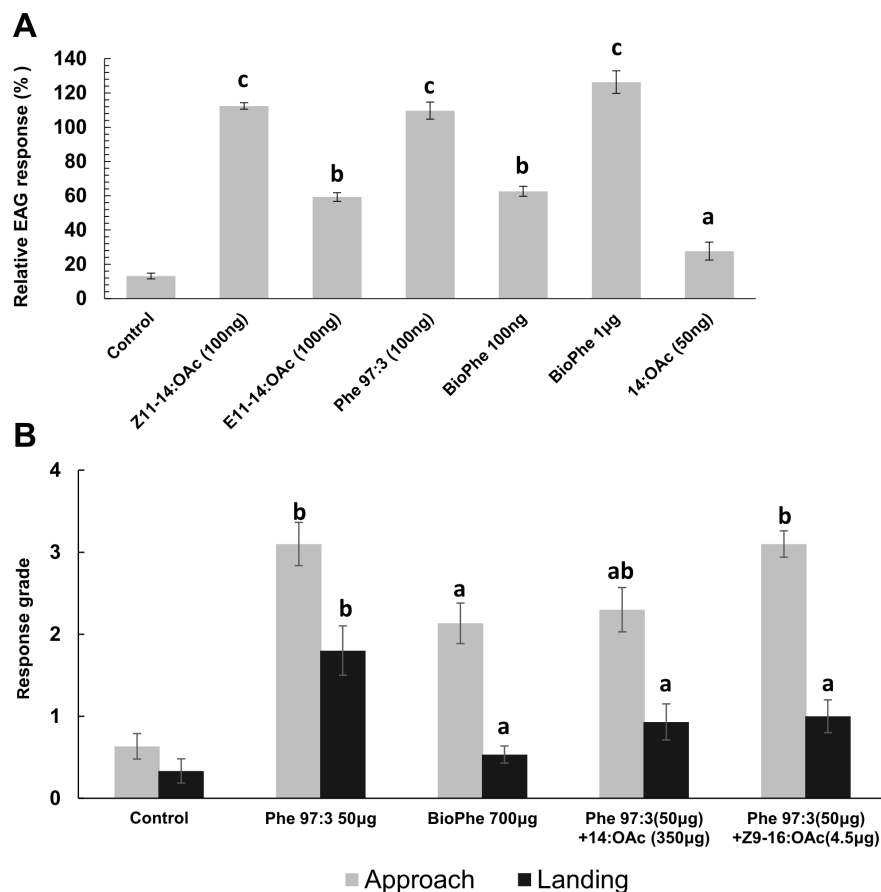


Figure 5. Performance of biologically-derived *O. nubilalis* sex pheromone. **A.** Electrophysiological responses of male *O. nubilalis* antennae to biologically-derived pheromone blend (BioPhe), standard compounds (*Z* 11-14:OAc, *E* 11-14:OAc and 14:OAc), and mixtures of the standard compounds. Response to 100 ng of *Z* 11-14:OAc was defined as 100%, and all responses normalized in % relative to this reference. Means followed by the same letter are not significantly different ($P > 0.5$, Tukey's studentized range [HSD] test, $F=51.650$, $df=5$, $P=0.000$) **B.** Behavioral responses of *O. nubilalis* males to yeast-derived pheromone blend (BioPhe), standard compounds (*Z* 11-14:OAc, *E* 11-14:OAc, 14:OAc) and mixtures of the standard compounds. Phe 97:3 represents pheromone composition originally found in *O. nubilalis* Z-race and is generated from standard compounds *Z* 11-14:OAc, *E* 11-14:OAc in ratio 97:3. Means followed by the same letter are not significantly different ($P > 0.05$, Tukey's studentized range [HSD] test). Approach, $F=4.579$ $df=3$, $P=0.005$, Landing, $F=5.899$, $df=3$, $P=0.001$)