Insecticide Assay 0-10mM

**Don’t see much point. Profiling an effect that isn’t relevant in realistic field settings.**

**For looking at the effect of the solvent. Again, doesn’t really matter because although DMSO is a co-formulant, it isn’t the only one. Therefore, studying its effects in isolation has limited use.**

Justification

Existing studies have only investigated the effects of nicotinic acetylcholine receptor (nAchR) targeting insecticide formulations in yeast, not the active ingredients alone 1,2. My initial range finding experiment suggested a differential response between cyanoimines and no effect for nitroguanidines or butenolides. The proposed experiment will combine testing the effects of solvent alone (DMSO, common additive in insecticide formulations) and in combination with insecticide. This will help explain if previously observed effects of neonicotinoid formulations in yeast were driven by additives or the active ingredients. Furthermore, confirming previously determined thiacloprid sensitivity in yeast will display off target effects, independent of nAchR interactions, in a eukaryotic organism.

The main criticism of this experiment is its relevance to field settings due to the high concentrations used. In a recent paper, 350-2800 ppm was stated as the range for soil application of imidacloprid in agricultural settings3,4. 2800ppm equates to 10.95mM for imidacloprid. This was, however, for pot not field application. The highest field-based soil application rate was for apple trees as 6ml/l, which is equal to 1200 ppm, or 4.69 mM. These recommended soil application rates show that soil microorganisms will be exposed to concentrations similar to those outlined in the proposed experiment. Any uncovered sensitivity will highlight the potential risk posed to soil fungi. The results from the range finding experiment (up to 5mM) showed no effect of imidacloprid alone. However, the additives (DMSO) could be having an effect. The proposed experiment will clarify this.

Another reason for using high insecticide concentrations is yeast’s efficient detoxification systems. These include a battery of tightly regulated ATP binding cassette (ABC) drug efflux pumps in the plasma membrane, which control the effective excretion of multitudinous structurally diverse molecules5. Indeed, overexpression of a particular ABC plasma membrane transporter, Pdr5p, leads to pleiotropic drug resistance (PDR), and this transporter is highly constitutively expressed during the logarithmic growth phase6. Due to these effective detoxification mechanisms, intracellular insecticide concentrations are likely much lower than in the surrounding extracellular medium.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | 0 | 2 | 4 | 6 | 8 | 10 |
| control |  |  |  |  |  |  |
| control+solvent |  | ref |  |  |  |  |
| thiacloprid |  |  |  |  |  |  |
| acetamiprid |  |  |  |  |  |  |
| imidacloprid |  |  |  |  |  |  |
| clothianidin |  |  |  |  |  |  |
| flupyradifurone |  |  |  |  |  |  |

* Insecticides – thiacloprid, acetamiprid, imidacloprid, clothianidin and flupyradifurone.
* 0, 0+solvent, 2, 4, 6, 8, 10mM. This means 2% DMSO.
* 10 replicates for each concentration above for each insecticide.
* Compare interaction between control+solvent (no insecticide) and concentration (no interaction) to interaction between insecticide and concentration (potential interaction).
* Also allows me to compare control to control+solvent.
* The insecticide 0mM observations are the same as control+solvent.
* If I only had control+solvent as part of insecticide (as the 0mM concentration) it raises two issues: wouldn’t be able to compare control to control+solvent and wouldn’t be able to compare the interaction of concentration and insecticide (insecticide) to the interaction of concentration and control+solvent (no insecticide).
* Control (no solvent) won’t be looked at regarding interaction with concentration. Still preferable to have same number of replicates for control and control+solvent to enable comparison.

Media Preparation

Follow Thorpe Lab standard protocol. To make 500ml yeast, peptone, dextrose (YPD) media:

**350ml sterile H2O**

**5g Yeast Extract**

**10g Peptone**

**100ml H2O**

**Once mixed autoclave**

**After autoclaving add 50ml of filter sterilised glucose**

Adding the glucose afterwards avoids the glucose degradation during autoclaving, which leads to variable growth mediums.

Yeast Overnight Culture

For replicates performed on different days to be comparable yeast cells have to be harvested from the same growth phase. Cells will be harvested from overnight cultures in mid-log phase, which is 0.4-0.6 OD on the Thorpelab spectrophotometer. The stock wild-type yeast strain, BY4741, is stored as colonies on agar plates at 4oC. Innoculate 5ml of YPD culture with 1 yeast colony. Mix the cells and perform x5 serial dilution five times (i.e. 5x, 25x, 125x, 625x and 3125x dilutions). Place these overnight cultures in the orbital shaking incubator (225rpm) overnight at 30oC. **Begin and end process at the same time every day (1100, 1300).** By the following morning, I expect that one of the serial dilutions will be in log phase (between 0.4-0.6 OD) (x125). Use log phase overnight culture for subsequent experiments. To increase cover also perform x10, x20, x50, x75 and x100 dilutions. If an overnight culture isn’t within the 0.4-0.6 OD range, then the experiment has to start again. **Label each tube with dilution factor and date**

x1 = 10ml media + 2 colonies

x5 = 8ml media + 2ml x1

x10 = 2.5ml media + 2.5ml x5

x20 = 3.75ml media + 1.25ml x5

x25 = 8ml media + 2ml x5

x50 = 2.5ml media + 2.5ml x25

x75 = 3.35ml media + 1.65ml x25

x100 = 3.75ml media + 1.25ml x25

x125 = 4ml media + 1ml x25

Dilute this overnight culture down to 4ml 0.05 OD solution.

Insecticide Assay

* Final Well Volume = 200µl
* Plate reader settings: 30°C Number of cycles = 193, number of flashes per well and cycle = 22, cycle time = 300s, shaking options = double orbital, 200rpm, 272s, before each cycle.
* Pipetting performed in sterile hood.
* Create **4ml** 0.05 OD yeast culture stock and dilute down to final theoretical 0.01 OD.
* 2% final DMSO concentration for all wells.
* P200 and P20 pipette.
* 10 replicates. Two from each day.
* 5 insecticides and two controls. One row per treatment (+ blank). Randomised within row.
* Use a heated Eppendorf block when creating stock solutions to combat solubility issue with Thiacloprid. Add the media, allow to heat up then add the master stock solution. Hopefully will prevent precipitation.
* Slide master stocks down the side of the Eppendorf (should do this anyway). Aids dissolution.
* Create plates as usual. Before adding 0.05 yeast OD stock, put plate on Eppendorf block for 15 minutes to allow it to heat up to 30°C and mix. Whilst this is going on get the yeast out of the orbital shaker and dilute down to 0.05 OD. **Keep a culture tube of media in the shaker too so also at 30°C.**
* Heat master solutions to 37°C (incubator) before making working solutions.
* Use multichannel to add 0.05OD yeast. Practice with this.
* Make stock solutions **first**, then DMSO, then yeast culture **last**.
* When collecting results, check 3 random wells for contamination (microscope in Thorpe lab). Blanks also show this but better to be safe.

Plate Design

Each row on the plate will be assigned a treatment (or blanks). The row allocation will be randomised so the treatments can’t be allocated to the same row twice on different days. Within a row there will be two replicates from each of the six concentrations per day (10 replicates in total). These will be randomised.

Stock Solution

* **Make 700µl of 20mM stock solution** from 500mM stock solution:
  + 28µl 500mM stock solution + 672µl media. **No! Precipitates out when added to media for Thiacloprid and Imidacloprid. Thiacloprid precipitating out at 10mM now too!**

Well Construction

* **10mM final well concentration:** 
  + 100µl 20mM stock solution + 60µl media + 40µl yeast
* **8mM final well concentration:** 
  + 80µl 20mM stock solution + 0.8µl DMSO + 79.2µl media + 40µl yeast
* **6mM final well concentration:**
  + 60µl 20mM stock solution + 1.6µl DMSO + 98.4 µl media + 40µl yeast
* **4mM final well concentration:**
  + 40µl 20mM stock solution + 2.4µl DMSO + 117.6µl media + 40µl yeast
* **2mM final well concentration:**
  + 20µl 20mM stock solution + 3.2µl DMSO + 136.8µl media + 40µl yeast
* **0mM final well concentration:**
  + 4µl DMSO + 156µl media + 40µl yeast stock
* **control + solvent:**
  + 4µl DMSO + 156µl media + 40µl yeast stock
* **control:**
  + 160µl media + 40µl yeast stock
* **blank:**
  + 200µl media

Correct Pipetting Technique

1. Aspiration
   1. Attach tip.
   2. Press down to first setting.
   3. Keeping the pipette vertical, insert tip into fluid just enough to be submerged but no more. Pre-wet tip 2-3 times. Eject fluid onto side of Eppendorf at 25-40° angle.
   4. Press down to first setting, insert vertical tip and aspirate sample fluid.
2. Dispensing
   1. Angle pipette at 20-45°, place tip on vessel wall and press down to first setting, then second setting.
   2. Eject tip.

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