CRDF Antimicrobial Strategy: GRAS-Like Compounds

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Overview

- The InnoCentive™ assay identified two naturally occurring broad spectrum antimicrobial plant essential oils, p-cymene and carvacrol, that have shown activity against C.Las in both the graft and L.crescens assays. The graft-based assay also measured phytotoxicity, and both compounds showed acceptably low levels with the concentrations tested.
- If one or both of these could be formulated for phloem delivery, they could be an attractive alternative to antibiotics, surfactants, and other powerful (and regulated) antimicrobial compounds.
- Both p-cymene and carvacrol are naturally occurring terpenoid essential oils, generally regarded as safe (GRAS) with a number of botanical sources, including oregano and thyme.
- Oil of thyme is on the 25(b) Minimum Risk Pesticide List and has a tolerance exemption, which means that a formulation of oil of thyme with inert compounds from an EPA-approved list would mean that no EPA approvals would be required. Usage would still be subject to state-level review, and the St ate of Florida would need to approve usage.
- Sponsored research is underway to formulate and deliver compounds that qualify for the GRAS-like status.
- The appeal of this category is the potential for more rapid deployment through a reduced commercialization and regulatory pathway if the laboratory evidence for efficacy of these materials is translated into the field.

InnoCentive™ Assays

- Graft-based assay
  - Infected scion soaked in test solution and grafted onto uninfected rootstock, follow by PCR
  - Slow, low-throughput
  - Evidence of efficacy *in planta* and first look at phyto-toxicity
  - Open contest with InnoCentive™ promotion
- *Liberibacter crescens* culture-based assay
  - Much faster, higher-throughput followed by *in planta* confirmation on *CLas*

About P-Cymene and Carvacrol

P-Cymene

- A naturally occurring aromatic organic compound, classified as an alkybenzene related to a monoterpene.
- Its structure consists of a benzene ring para-substituted with a methyl group and an isopropyl group.
- P-cymene is the only naturally occurring cymene isomer that includes o-Cymene (alkyl groups are ortho-substituted) and m-cymene (alkyl groups are meta substituted).
- P-Cymene is insoluble in water, but miscible with ethanol and diethyl ether.
- It is a constituent of a number of essential oils, most commonly the oil of cumin, thyme and oregano. Significant amounts are formed in the sulfite pulping process from wood terpenes. It is available in commercial quantities.

Carvacrol

- A naturally occurring organic compound, classified as a monoterpenoid phenol. It has a characteristic pungent, warm odor of oregano.
- It is present in the essential oil of oregano, oil of thyme, oil obtained from black cumin, marjoram, Dittany of Crete, and wild bergamot. It is also found in tequila.
- Carvacrol concentrations can be found ranging from 30 to 87% in traditionally distilled Oregano oils.
- Essential oil of thyme subspecies contain between 5% and 75% of carvacrol, while marjoram and Dittany of Crete are rich in carvacrol, with 60 to 80%.
- Carvacrol is known to inhibit growth of several bacterial strains, has low toxicity, and is widely used as a food additive to prevent bacterial contamination.
- There are few identified long-term genotoxic risks. The cytotoxic effect can make it an effective antiseptic and antimicrobial agent, and it has also been found to show antioxidant activity. It is available in commercial quantities.

Antimicrobial Activities of Essential Oils

Oil of Oregano

- More than 40 different varieties (chemotypes) of oregano
- Contains a number of compounds, including carvacrol, thymol, p-cymene, cis-ocimene, caryophyllene and linalool.
- The CT Carvacrol chemotype for oregano (Oreganum vulgare) is high in carvacrol (60 to 75%) and low in thymol (less than 5%) and is most used for human and animal medicinal purposes.
- Extracted from the dried, flowering tops of the herb by steam distillation.
- Typically delivered orally with a carrier oil (e.g. olive) for oral use, also can be applied topically.
- Also has been used in animals, among other things, as an alternative to antibiotics.

Thyme Oil

- Thymus vulgaris
- The main chemical components are a-thujone, a-pinene, camphene, b-pinene, p-cymene, a-terpinene, linalool, borneol, b-caryophyllene, thymol and carvacrol.
- There are various chemotypes of thymus vulgaris which have differing chemical constituents. CT thymol contains strong antiseptic properties and is 60 to 70% thymol. CT carvacrol also has strong antiseptic properties and is 30 to 80% carvacrol, depending on time of harvest.
- Extracted from the dried, flowering tops of the herb by steam distillation
- Sold over the counter for human consumption for its antibacterial and anti-fungal properties
- Delivered via vaporizer, orally and topically, typically delivered with a carrier oil (e.g. olive) for oral use

Minimum Risk Pesticides

- Minimum risk pesticides are a special class of pesticides that are not subject to federal registration requirements because their ingredients, both active and inert, are demonstrably safe for the intended use.
- To qualify for the FIFRA 25(b) exemption all five of the following conditions must be met.

  1. The product must contain ONLY active ingredients that are on the list.

  2. The product must contain ONLY those inert ingredients that have been classified as list 4A "Inert Ingredients of Minimal Concern"

  3. All of the ingredients (both active and inert) must be listed on the label. The active ingredients by name and percentage by weight; each inert must be listed by name.

  4. The label cannot include any false or misleading statements and claims for protecting human or public health are prohibited

  5. Labels may not bear claims to control rodent, insect or microbial pests in a way that links the pests with any specific disease
- Additionally, some of the active ingredients will require pesticide residue tolerances be established.
- There are thirty-one (31) active ingredients exempted under 25(b), including oil of thyme, which contains p-cymene and carvacrol, as well as thyme oil.

Minimum Risk Pesticides: Active Ingredients

Castor oil	Garlic and garlic oil*	Putrescent whole egg solids
Cedar oil	Geraniol*	Rosemary and rosemary oil*
Cinnamon and cinnamon oil*	Geranium oil	Sesame and sesame oil*
Citric Acid*	Lauryl sulfate	Sodium chloride*
Citronella and citronella oil	Lemongrass oil	Sodium lauryl sulfate
Cloves and clove oil*	Linseed oil	Soybean oil
Corn gluten meal*	Malic acid	**Thyme and thyme oil***
Corn oil*	Mint and mint oil	White pepper
Cottonseed oil*	Peppermint and peppermint oil*	Zinc metal strips
Dried blood	2-Phenethyl propionate	
Eugenol	Potassium sorbate*	

* Indicates exempt from pesticide residue tolerance requirements
Bold type indicates contains carvacrol and/or p-cymene

Minimum Risk Pesticides: Inert Ingredients

- Minimum risk pesticides may only contain the minimal risk inert ingredients noted in List 4A. A description and list can be found at:

  http://www.epa.gov/oppbppd1/biopesticides/regtools/25b/25b-inerts.htm

  http://www.epa.gov/opprd001/inerts/section25b_inerts.pdf
- Minimum risk pesticide producers may use List 4A inert ingredients in whatever amounts they believe will result in an effective product when combined with 25(b) active ingredients. Inert ingredients are defined as all ingredients that are not active ingredients. Active ingredients are the ingredients that kill, repel, or mitigate the pest.
- EPA's determination that an inert ingredient poses minimal risk is based on the following:
  - The Agency's recognition of the overall safety of the substance (such as very low toxicity or being practically non-toxic)
  - Consideration of the widely available information on the substance's known properties
  - A history of safe use under reasonable circumstances
- Minimum risk pesticide labels must include the name of each inert ingredient, as well as listing the name and percentage by weight of active pesticidal ingredients.

Example of a Minimum Risk Pesticide Label

- Product Name: Pro-Max
- Company: Bio-Humanetics
- Use: Broad spectrum soil fungicide and nematicide for use in Agronomic crops, vegetables, tree crops, turf and ornamentals.
- Active Ingredients: Thyme oil: 3.5%
- Inert Ingredients (per label):
  - Glycerin
  - Molasses
  - Water
- MSDS description
  - Thyme oil
    - Thymol
  - Glycerin
  - Diammonium phosphate
  - Proprietary enzyme
- Represented on label as exempt from EPA registration, meets requirements of Natural Organics Program.
- http://humagroturf.com/tag/promax/

Example of Carvacrol-Based Product

- Company: Chengdu Newsun Crop Science Co, Ltd
- Company Location: Sichuan China
- Product: Biological Fungicide Carvacrol 0.5% AS
- Extracted from plants
- Used to treat grey mold in vegetables
- Inert ingredients not listed.
- http://www.cdxzy.com/en/proC/201303/182.html

Example of EPA Registered Oil of Oregano-Based Product

- EPA registered MossBuster as a herbicide in 2011 to control moss and other bryophytes (algae, lichen and fungi).
- Product label identifies active ingredients a 1% certified organic essential oil, 99% other ingredients (not identified).
- EPA registration information indicates the essential oil is Oil of Oregano.
- Proprietary formulation. Patent mentions oil of oregano, hydrocolloid ticgum, gum arabic, arabanoglactin
- Label claims it contains no bleach or acids and is biodegradable.
- Company is Wes Chem, based in Iowa.
- http://www.weschem.ie/mossbuster.php

Other Essential Oil Examples

Product	Company	Ingredients
Organic Fungicide Patented Formulas http://www.ecosmart.com/wp-content/docs/msds/garden-fungicide.pdf	EcoSmart Not Registered	Rosemary Oil 0.24% Sodium Lauryl Sulfate 0.2% Water, Wintergreen oil, mineral oil, lecithins 99.56%

CRDF Formulation Alternatives

- Evaluate existing commercially available products containing thyme oil, oil of oregano, or their components such as thymol, carvacrol and p-cymene.
- Add thyme oil, oil of oregano or its components to an existing commercially available product
- Formulate a new product(s) based on plant essential oils and inerts that is optimized for efficacy against HLB
- Screen all of the reasonably available (not blood and eggs) 25(b) compounds for activity

Formulation Issues

- Commercial application of oil of oregano, oil of thyme or its constituents such as thymol, carvacrol and p-cymene, requires a formulation approach that protects them from degradation and evaporation, while at the same time allows for sustained release and delivery to the phloem in a way that does not cause phytotoxicity.
- For GRAS-like compounds, CRDF is continuing to investigate and test “nano-emulsions” and other formulations through sponsored research at the UF Particle Engineering Research Center with intent to use toll manufacturing, as needed, to scale up candidates for field trials. We can follow-up on commercial collaborations as appropriate.
- The evaluation includes encapsulating the essential in various concentrations in oil droplets of nano-emulsions, prepared by high pressure homogenization and stabilized by different emulsifiers.
- These nano-emulsions will then need to be formulated with other ingredients or added to existing products in a way to maximize antimicrobial efficacy against HLB.

Summary

- P-cymene and carvacrol have demonstrated some efficacy against HLB in screening.
- These are found, along with other ingredients, in oil of oregano and thyme oil
- Thyme oil is on the 25(b) minimum risk pesticide list
- Most GRAS product labels do not contain detailed information about ingredients beyond active ingredient.
- CRDF plans to explore three different formulation approaches: use existing products, add the essential oil to an existing product, or formulate a new product optimized against HLB
- Because of the regulatory advantages of the 25(b) category, a thorough screen for antibacterial activity is warranted.
- Ongoing work with UF Particle Engineering Research Center is looking at nano-emulsion approaches to formulation
- For the formulation to be effective, it must be able to reach the phloem of the HLB-infected tree to attack the CLas bacterium.
- Minimum regulatory requirements and reduced time to market are major criteria in developing formulation alternatives.