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New 'stimuli-enriched' laboratory bioassay used to identify improved botanical repellent treatment, Lem-ocimum, to control the stored-grain pest *Tribolium castaneum*

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ABSTRACT

A laboratory study of *Tribolium castaneum*, a major pest of stored grain, was conducted to develop a more efficient and effective 'choice' bioassay for identification of new repellent botanical treatments. Standard bioassays to test the repellency of candidate plants include pit-fall traps and open arena choice tests, environments lacking in some of the most important natural stimuli that guide the movement of food-searching beetles, e.g., 1) materials they can burrow through, which stimulate 'positive thigmotaxis', 2) a range of light and dark areas, which stimulate 'negative phototaxis' and 3) three-dimensional habitats, which stimulate 'positive geotaxis.' The lack of these stimuli can lead to two common problems; 'low efficiency' (high proportion of beetles remain in the area that surrounds treatments without making a 'choice'), and 'low efficacy' (high variability in proportions found in control and treated samples). The new 'stimuli-enriched' bioassay, which included all three of the above stimuli, was significantly more efficient ($P < 0.0001$) and effective than three standard bioassays. The stimuli-enriched bioassay was used to compare the repellency of four candidate plants; *Ocimum basilicum* (Sweet Basil) and *Cymbopogon nardus* (Lemongrass) were significantly more repellent than *Vernonia amygdalina* or *Nauclea diderrichii* (Tukey Contrasts; $P < 0.01$). A novel method of applying repellent material (a paste of repellent plant is applied between the layers of double bagged grain) was tested on the most promising repellent plants materials; a combination of *C. nardus* and *O. basilicum* ('Lem-ocimum') at 0.5% w/w of each was significantly more effective than *O. basilicum* on its own (Tukey Contrasts; $P < 0.05$). These results show that the stimuli-enriched bioassay provides more consistent and accurate assessments than the standard bioassays of the repellency of candidate botanicals, and that Lem-ocimum treated double-bags are a promising new method of protecting sorghum from *T. castaneum*.

1. Introduction

Monitoring and control of pest insects is often based on controlling their behaviour by presenting semio-chemicals (chemical attractants or repellents) to trap, kill or repel target species. Laboratory-based bioassays have been an important tool for testing the behavioural responses of target species to candidate compounds under controlled semi-natural environmental conditions prior to field testing under natural conditions (Robertson et al., 2007); large numbers of insects can be tested against a wide range of chemicals and doses relatively quickly. In particular, bioassays have played a key role in determining the efficacy of repellent plant materials and in identifying their active ingredients against storage crop insect pests (Morgan et al., 1998; Lale and Yusuf, 2000; Stefanazzi et al., 2011). However, the strength of response, which determines the efficacy of a bioassay, depends on the quality of the environmental stimuli present in the bioassay. Many standard bioassays test whether a material is an attractant or repellent by measuring the insect's response (positive or negative 'chemotaxis') to volatile chemicals emanating from the material and carried by a moving air current (Campbell, 2012). The movement of insects in their natural environments, however, is also controlled by responses to a wide range of stimuli; e.g., 'phototaxis'

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causes movement toward or away from light (Reza and Parween, 2006), 'geotaxis' causes movement up or down in response to gravity (Cox and Collins, 2002; Jiang et al., 2006) and 'thigmotaxis' causes movement along pathways that maximise the area of their bodies in contact with surfaces (Kennedy, 1986). All of these stimuli-driven responses help beetles locate a food source and avoid contact with toxic chemicals, while keeping them in a protected environment. Hence, bioassays should incorporate the main stimuli insects respond to in specific environments, so that the outcome of the bioassay reflects what is likely to occur in the field. Otherwise, a stimulus-poor bioassay can interfere with the natural searching behaviour of beetles, and, therefore, reduce the reliability of the bioassay outcome.

It has been observed that a major problem with *Tribolium castaneum* (Herbst) bioassays is that significant proportions of the beetles spend most of their time walking around the edges of the arena where they can be in contact with the floor and a wall, infrequently moving across the open area of the arena (Surtees, 1964; Yinon and Shulov, 1969; Campbell and Hagstrum, 2002; Olsson et al., 2006; Duehl et al., 2011; Campbell, 2012). A study by Campbell and Hagstrum (2002) of the behaviour of *T. castaneum* in a bioassay arena found that they moved across a bioassay arena more frequently if a network of walls was present throughout the arena. We tested the hypothesis that this is due, at least in part, to the strength of their response to thigmotactic cues; i.e that beetles prefer to maintain contact with substrates over as much of their bodies as possible, presumably to protect themselves from desiccation and from detection by predators (Romero et al., 2010).

In the study presented here, a new bioassay was designed to take into account phototaxis, geotaxis and a rarely considered response, thigmotaxis, with the aim of identifying promising repellents with greater efficiency and efficacy than three standard bioassays (long-drop pitfall, open arena and open arena with shallow pits). The new, stimuli-enriched bioassay was tested against these three standard bioassays to compare the strength of response of *T. castaneum* to sorghum grain treated with a known repellent, methyl salicylate.

The bioassay was also used to compare the response of *T. castaneum* to four commonly used repellent plants; Sweet basil (*Ocimum basilicum* (L.)), Lemongrass (*Cymbopogon nardus* (L.)), Bitter leaf (*Vernonia amygdalina*) (Delile), Yellow tree (*Nauclea diderrichii*) (De Wild & T. Durand) Merrill and a combination of the two most repellent plants; Lemongrass (*C. nardus*) and Sweet basil (*O. basilicum*), hereafter referred to as 'Lem-ocimum', plants that are grown in the area of the field experiments in a laboratory in Kebbi, Nigeria. These species were chosen because there is evidence in the literature (Asawalam and Hassanali, 2006; Musa et al., 2009; Mishra et al., 2012) that they are repellent to a range of stored crop pests. However, information on the efficacy of these plant materials on *T. castaneum* infesting sorghum is limited, and no published information was found on the efficacy of dried powder of these plants on *T. castaneum*.

Finally, the bioassay was used to assess the efficacy of a new method for protecting grain from *T. castaneum* infestations developed by Utono (2013) in response to evidence that the conventional method of mixing repellent botanicals with grain storing in bags does not give optimum protection, as practiced by farmers in study area of Utono (2013) and the present study area (Kebbi state, Nigeria) and described by Koona et al. (2007) for use against cowpea beetles. Mixing repellent plant materials with stored grain does not reduce the ability of insects to penetrate into the bags, the repellent plant material is dispersed at a relatively low density throughout the grain, and it is time-consuming to remove the repellent plant material before preparing the grain for food or for selling (Utono, 2013). Therefore, Utono (2013) and developed and field-tested a 'repellent-treated double-bag' method for protecting sorghum grain based on the results of stimuli-enriched bioassays presented here. A paste of dried Lem-ocimum is applied between the layers of a double bag, and just pure grain is placed within the inner bag, with the aim of concentrating the repellent plant material in a layer surrounding the grain, and increasing the physical impedance of beetles from moving through two layers of bag to reach the grain.

The main aims of this study were to design a more efficient and effective bioassay to identify from a range of plant materials, the plants and doses with the most promising repellent effects to protect stored grain from *T. castaneum*, and to evaluate the effectiveness of a new method for applying repellent plant material to stored grain; double bags treated with a low dose of a repellent plant material to protect grain within the inner bag grain from beetle infestations.

2. Materials and methods

2.1. Experiment 1: Development of a new 'stimuli-enriched' bioassay

A new bioassay apparatus was designed with the aim of increasing the efficiency and efficacy with which the assay measures the response of *T. castaneum* to test stimuli, as compared to standard bioassays, by incorporating a greater number of environmental stimuli that if encountered could mediate the behaviour of beetles when they search for food. In this case, an '**efficient**' bioassay is defined as resulting in a high proportion of test beetles caught in either treated or untreated grain, with few wandering around in the rest of the assay arena, and an '**effective**' bioassay is defined as producing the clearest difference in response to the control and treatment, i.e. the least variable results and the greatest difference in the proportions of beetles caught in the untreated and treated grain for a given dose. To assess the relative improvement of the new bioassay, the response of beetles to a standard dose of a known repellent, methyl-salicylate, was compared using three standard bioassays (pitfall trap and two versions of an open arena choice test) and the new bioassay.

2.1.1. Standard bioassays

2.1.1.1. Pitfall traps. 'Pitfall' type choice traps rely on the movement of beetles throughout the trap arena to bring them within detectable range of the test material, whereupon they fall into the trap if the test material is a suitable attractant or avoid the trap if it is a repellent, The pitfall trap used for this study consists of a petri dish (9 cm in diameter, Alpha Laboratories UK) with two holes, placed equidistant to the sides of the dish and each other, and each hole is fitted with an eppendorf tube (1.5 ml, Alpha Laboratories UK) with the bottom cut off, such that the tops of the eppendorf tubes are level with the floor of the petri dish. Each of the eppendorf tubes is inserted into one of two centrifuge tubes (15 ml, Alpha Laboratories UK) underneath the petri dish (Fig. 1A). One centrifuge tube contains the treated test grain sample and the other contains the control (untreated) test sample. This apparatus allows odour from the tubes to emanate upwards into the petri dish, and beetles released in the centre of the petri dish 'choose' between the two odours.

2.1.1.2. Open arena choice test. This bioassay apparatus consists of a large open tray arena (58 cm long × 39 cm wide × 8.5 cm deep) in which beetles can move around freely and make a choice between the control and treated grain samples, which are contained in netting bags (mesh size = 1.5 × 1.5 mm, bag = 8 × 8 cm) placed on the floor at either end of the rectangular tray (Fig. 1B). These bags