Trace Rice

Testing trials of the solutions selected for prevention of

(i) insect infestation

13 August 2024

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TECHNICAL REFERENCES

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07/08/2024	INIAV	V2	Text revision and addition of an executive summary
13/08/2024	INIAV	V3	Final Version approved by project coordinator

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EXECUTIVE SUMMARY

The urgency to find innovative solutions for preventing insect infestations in rice storage has led to the exploration of essential oils, with Limonene, Eugenol, and Eucalyptol showing promise in repelling *Sitophilus* species. While plastic packaging currently provides protection against infestations in retail settings, there is a pressing need to develop biodegradable materials that also prevent insect contamination.

Preventing insect damage in rice necessitates the thorough cleaning of facilities as a first step, due to the near inevitability of storage infestations. Additionally, alternative methods to prevent weevil infestations originating in the field are crucial.

The study revealed that CO_2 levels increase significantly throughout the insects' life cycle, a change not observed in non-infested rice, indicating CO_2 levels as a reliable marker for hidden infestations. The qRT-PCR method shows potential; however, further tests with blank samples and improvements are needed to confirm its industrial applicability.

The TRACE-RICE project aimed to find chemical-free solutions for preventing egg hatching and controlling insect infestations in stored rice. The trials suggest that these innovative methods can effectively maintain rice quality and reduce reliance on chemical treatments. Prevention techniques, such as using the O_2/CO_2 portable sensor for detecting hidden infestations, proved valuable. The study identified the impregnation of rice with essential oils Limonene, Eugenol, and Eucalyptol, along with UV radiation application, as promising solutions for further validation and replication.

1. Introduction

1.1 Statement of Problem

Every year, tons of rice (*Oryza sativa* L.) are rejected due to insect infestations, posing a significant problem in many countries. This issue has always affected Portuguese companies, but with Portugal rapid growth as a rice export country, the potential financial impact has increased in the last years. Rice is one of the most consumed cereals worldwide (FAO, 2019), making it essential to develop solutions to minimize losses and ensure quality and safety for consumers, as well as sustainability in the supply chain.

Current mitigation strategies rely on synthetic chemicals that do not definitively eliminate infestations and whose use is increasingly questioned by consumers and regulators. Moreover, insect species are developing resistance to these chemicals (Lee et al., 2003). *Sitophilus* species are the primary agents of these infestations, capable of destroying grains and laying eggs that develop inside them until the adult insect stage. Since most of *Sitophilus* life cycle occurs inside the grains, detecting hidden infestations is a priority for effective prevention.

The search for innovative solutions to prevent insect infestations is urgent, and the application of essential oils shows promise. However, their repellent activity against *Sitophilus* species needs to be thoroughly assessed. Additionally, while plastic packaging material for rice in retail serves a protective function against infestations, there is a need to introduce biodegradable materials that can also prevent insect contamination.

1.1.1. Innovative Treatments

The use of innovative solutions to prevent insect infestations is highly promising, as it addresses both objectives of sustainable production and ensuring insect-free products. Laboratory-scale studies have reported encouraging results for various potential solutions, including the use of biopesticides in atmospheric applications or packaging films (De Fátima Silva et al., 2022; Goñi et al., 2017; Herrera et al., 2018; Hossain et al., 2019; Marsin & Muhamad, 2023; Wongphan et al., 2023), essential oils (Al-Harbi et al., 2021; Follett et al., 2014; Mishra et al., 2013; Zargari et al., 2022), and electromagnetic treatments such as microwaves (Zhao et al., 2007) and radiofrequency (Vearasilp et al., 2015). Additionally, combinations of various radiations have also been explored (Srivastava & Mishra, 2022). Proper implementation of these solutions could provide effective alternatives for protecting stored grain against pests (Herrera et al., 2018). However, their effectiveness on an industrial scale needs further evaluation to clearly define the cost-benefit proposition of these alternative methods proposed by researchers.

1.1.2. Hidden Infestation Detection

To minimize the cost and effort required with any type of solution, it is essential to develop effective methods for detecting hidden infestations. Current conventional techniques (ISO 6639-4, 1987) are outdated, necessitating the development of innovative tools for screening and controlling infestations. One promising alternative is the polymerase chain reaction (PCR), which has been used to detect specific genetic regions of target species and quantify early infestations (Negi et al., 2021; Solà et al., 2018).

The amount of carbon dioxide (CO_2) in rice storage is largely correlated with infestation due to the insects' respiration rate (Howe & Oxley, 1944). Therefore, measuring CO_2 levels can effectively estimate hidden infestations in rice, helping to prevent waste.

Monitoring hidden infestations and seeking efficient non-chemical solutions to mitigate insect problems will enhance the productivity and profitability of rice companies, benefiting both the environment and consumers.

2. Objective

The main objective of task 2.2 is to test the effectiveness of physical and biological-based solutions to eliminate the use of synthetic chemicals and ensure the eradication of insect infestations in stored rice. Accordingly, this report focuses on developing methods to control hidden infestations, testing innovative solutions to replace chemical substances, and evaluating sustainable packaging alternatives to plastic. The report covers the following areas: the reproduction cycle of insects; methods for detecting hidden infestations; tests on the repellent activity of essential oils; the efficiency of bioplastics in protecting rice against infestation and electromagnetic wave treatments.

3. Reproduction Cycle of Insects

The primary insects responsible for rice infestations are from the *Sitophilus* species, namely *Sitophilus oryzae* and *Sitophilus zeamais*, commonly known as weevils. To accurately address this issue, it is important to begin by understanding how these pests affect stored rice, the conditions under which they develop, and their life cycle.

3.1. Insects Identification

The original insects were obtained from the Entomology Laboratory at the Instituto Superior de Agronomia, Universidade de Lisboa, under the supervision of A. Mexia from the TRACE-RICE advisory board. Reproduction cycles were conducted under optimal development conditions (25±1°C and 70±5% RH) until the required number of insects for the trials was reached.

The two species of *Sitophilus*, as well as the sex of the insects, cannot be distinguished with the naked eye. Since this information is valuable for our results, identification is carried out by observing the genitalia under a microscope, as illustrated in Figure 1.

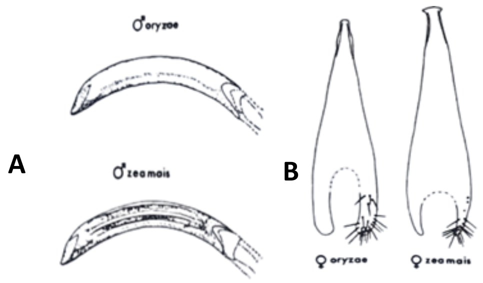

Figure 1 - A - aedeagus of male genitalia; B - sclerite of female genitalia.

For the identification process shown in Figure 2, the insects are boiled in 10% KOH at 100°C for approximately 20 minutes. They are then transferred through 70% ethanol and distilled water in a Petri dish. This procedure allows us to gather more detailed information for our results and analyse any differences in the responses of males and females. Since this identification can only be performed post-mortem, the process is conducted at the end of the tests.

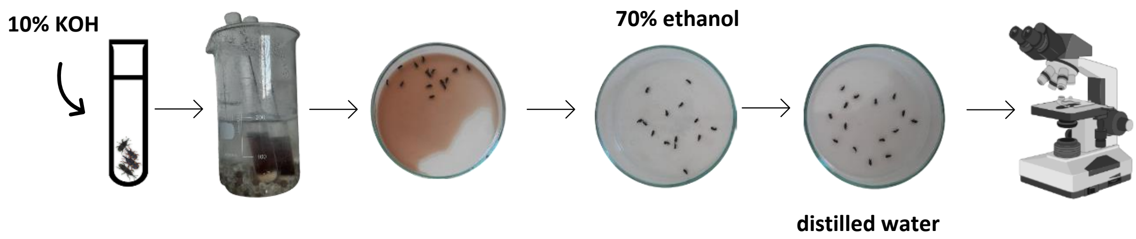

Figure 2 – Schematic representation of insect preparation for species and sex identification

3.2. Insect Collection and Identification

To date, we have obtained more than 5000 insects, of which approximately 460 have been collected and identified. As shown in Table 1, over 260 males and 200 females were identified.

Table 1 - Approximate Numbers of Reproduced, Identified, and Utilized Insects in the Study

Total insects	Total larvae/pupae	Identified insects		
~5000	~600	~460	*male*	*female*
			~260	~200

Larvae and pupae are also collected as they are essential for the hidden infestation determination method.

4. Methods for Detecting Hidden Infestations

Most of the *Sitophilus* life cycle occurs inside the grains. Adult insects create a hole in the grain to lay their eggs, sealing it with a gelatinous plug, which makes infected grains indistinguishable to the naked eye (Mesterházy et al., 2020). Therefore, it is crucial to develop methods that can detect hidden infestations and prevent the development of these insects.

4.1. CO_2 Sensor and qRT-PCR Methods

Hidden infestations can be detected using a portable sensor (Figure 3) that measures oxygen (O_2) and CO_2 levels, providing data on the insects' respiration rate. Additionally, we are developing a real-time quantitative polymerase chain reaction (qRT-PCR) method with specific primers for each species (*S. oryzae* and *S. zeamais*).

It is possible to detect hidden infestation using a portable sensor (Figure 3) that determines O_2 and CO_2 levels, obtaining data on the insects' respiration rate. Besides this, we are also developing a real-time quantitative polymerase chain reaction (qRT-PCR) method with specific primers for each species (*S. oryzae* and *S. zeamais*).

Daily measurements were taken using the O_2/CO_2 portable sensor (PBI Dansensor, CheckMate 9900) until adult insects were detected.

Figure 3 - O_2/CO_2 (%) portable sensor (PBI Dansensor, CheckMate 9900).

The qRT-PCR method is currently under development, with detection limits for both species being established. Initially, a DNA barcode approach was utilized, employing a standardized genomic region, the cytochrome c oxidase I (COI) gene, to design species-specific primers and probes, and assess their efficiency in amplifying target DNA. Subsequently, tests were conducted to determine the detection limits of the qRT-PCR method.

4.2. Results of Hidden Infestation Detection

Several tests using the portable sensor yielded very encouraging results. In these tests, 10 g of rice were infested with 10 insects each and left for 10 days. After this period, the insects were removed, and CO_2 levels were measured. Measurements of six replications were taken daily until adult insects were detected in all replicates. The results, shown in Figure 4, indicate a distinct increase in CO2 levels corresponding to the presence of insects. The data reveal a clear increase in CO_2 levels throughout the insects' life cycle, which was not observed in the control samples (non-infested rice). This suggests that CO_2 levels effectively indicate hidden infestations.

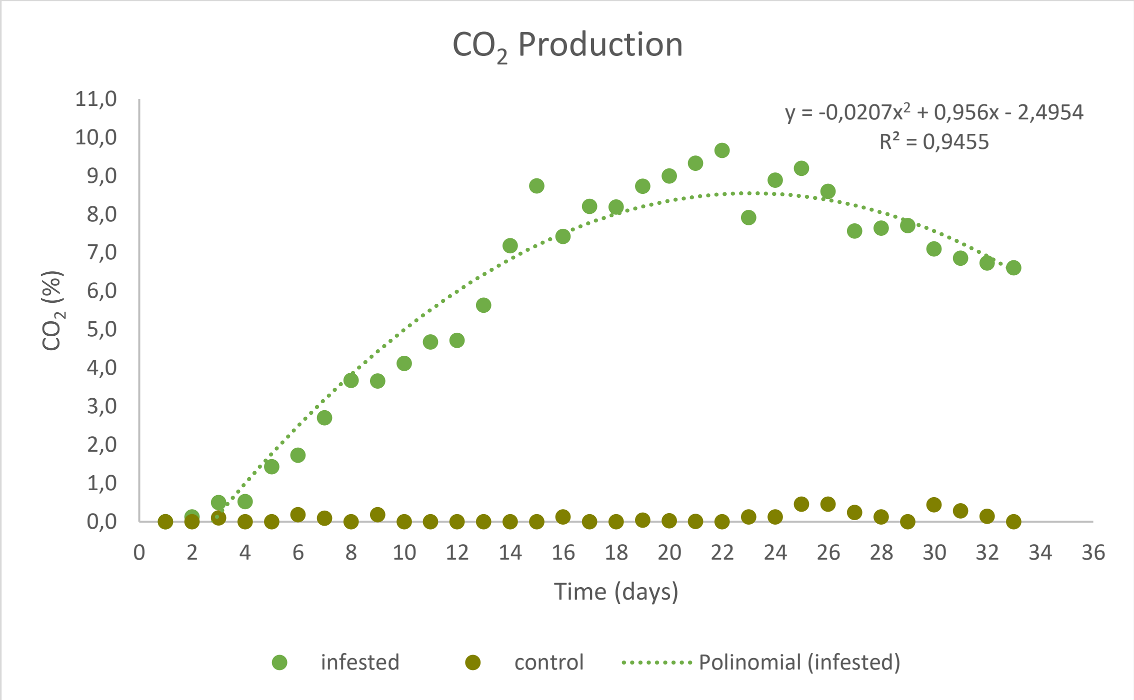

Figure 4 - Mean CO_2 levels (%) throughout the insect life cycle in infested rice samples. Note: Control corresponds to non-infested rice

Subsequently, the samples previously evaluated by the portable sensor were analysed using the qRT-PCR method. Repetitions of 10 grams of rice were again infested with 10 insects each and removed after 10 days. Once the predefined CO_2 levels of 0.2%, 0.5%, and 2% were reached, these samples were frozen and subsequently analysed by qRT-PCR. The results, shown in Table 2, confirmed the presence of DNA from both species in the samples that had previously been detected as infested.

Table 2 - Quantitative real-time PCR (qRT-PCR) results, in infested rice samples, with different levels of CO_2 (%).

CO2 (%)	qRT-PCR results
0.2	*S. zeamais* positive *S. oryzae* positive
0.5	*S. zeamais* positive *S. oryzae* positive
2	*S. zeamais* positive

The qRT-PCR method shows promise; however, all the tests conducted so far have yielded positive results. Additional data from blank samples (without any infestation) and further improvements are needed to determine its applicability in the industry.

5. Repellent Activity of Essential Oil Compounds

The application of essential oils for controlling infestations was reviewed in D2.1 and published (De Sousa et al., 2023). Subsequently, five food-grade essential oil compounds were selected for the trials: Eucalyptol, Eugenol, (S)-(-)-Limonene, L-Menthol, and Thymol.

5.1. Olfactometer Trials

The behaviour of *Sitophilus* species in response to different essential oils was evaluated using an olfactometer to estimate the repellent activity of these compounds. Trials were conducted with cotton disc embedded with three concentrations of each essential oil compound (200 µL, 100 µL, 50 µL) in a four-arm olfactometer, utilizing only two air inlets (Figure 5). The central arena of the olfactometer was divided into three regions (A, B, C), and each insect's behaviour was assessed over a 10-minute period. A cotton disc was placed in one of the air inlets, and an insect was introduced into the arena. For each olfactometer test, 15 insects were evaluated. The time spent in each region (near the compound, far from the compound, or in the central region) was then recorded. was recorded. The entire test was conducted in a dark environment under red light, and the insects were removed from their food substrate 24 hours before the test.

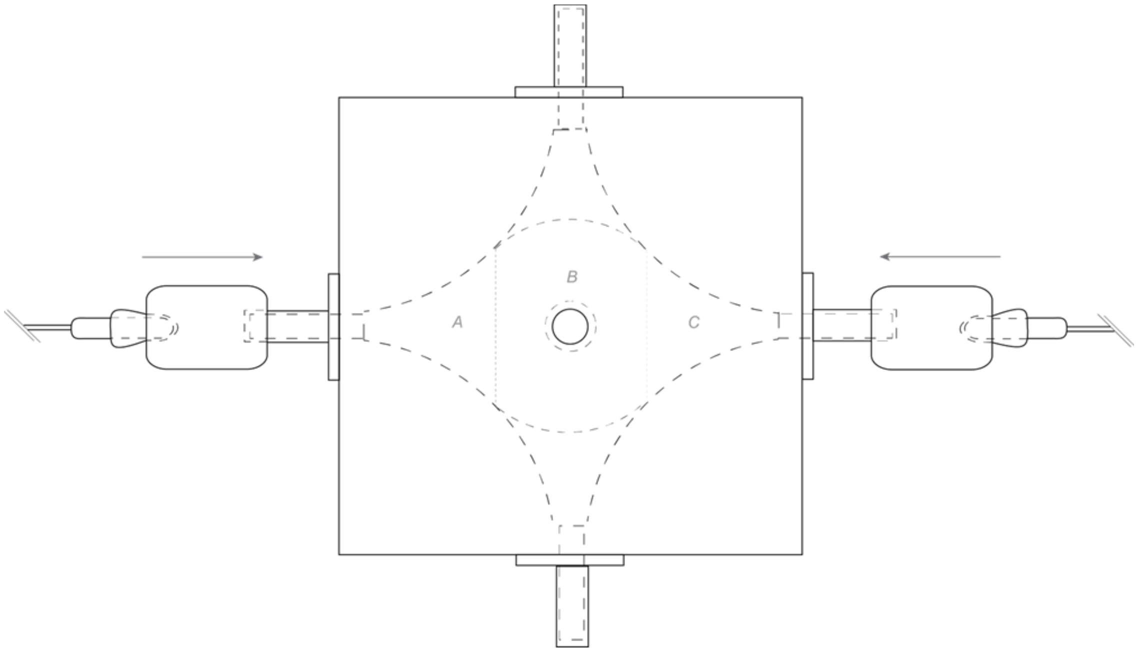

Figure 5 - Diagram of a four-arm olfactometer, illustrating the airflow route. (Note: A, B, and C correspond to the three regions of the central arena, used to analyse whether the insects were near or far from the stimulus source).

Prior to the essential oil trials and to ensure that the test conditions would not affect the insects' behaviour, two control tests were conducted. One test used a cotton disc with 200 mg of rice flour, while the other test used only a plain cotton disc, as the essential oil compounds were applied to cotton discs during the trials.

5.2. Results of Olfactometer Trials

The control results are shown in Table 3. No influence from the cotton disc was detected, as the insects spent roughly the same amount of time in all three regions, with no significant differences (p-value = 0.38571). As expected, a significant preference for the region of the olfactometer near the rice flour was observed (p-value = 0.001173).

Table 3 - Mean time (min.) spent by 15 insects in each location of the olfactometer: far, central or near using the cotton disc and rice flour in the air inlet. Different letters (a and b) indicate significant differences at p ≤ 0.05, as determined by the LSD test.

Mean Time (min.)			
Source	*near*	*central*	*far*
Cotton Disc	3,13±1,72[a]	3,90±1,64[a]	3,11±1,90[a]
Rice Flour (200 mg)	4,92±2,72[b]	2,95±1,89[a]	1,71±1,76[a]

Each essential oil compound was tested by evaluating the behaviour of 15 insects. Thymol and L-Menthol, which are solid at room temperature, were first dissolved in distilled water, 96% ethanol, and polysorbate 80 for testing. However, concerns arose regarding how the solubilization process might affect the efficacy of these compounds. Consequently, only the trials involving (S)-(-)-Limonene (L), Eugenol (EG), and Eucalyptol (EC) were selected for statistical analysis, as detailed in Table 4.

The results indicate that the insects spent more time in the central and distant areas of the olfactometer, avoiding the cotton discs with essential oil compounds, even at lower concentrations (50 µL). This behaviour suggests that these compounds have potential in preventing *Sitophilus* infestations. Currently, a panel-trained sensory analysis of rice samples treated with Limonene, Eugenol, and Eucalyptol is underway to further validate and potentially recommend these essential oils as effective solutions.

Table 4 - Mean time (min.) spent by 15 insects in each location of the olfactometer: far, central or near using the essential oils compounds in the air inlet. Different letters (a and b) indicate significant differences at p ≤ 0.05, as determined by the LSD test.

Mean Time (min.)			
Source	*near*	*central*	*far*
EC200	3,32±2,80[a]	2,92±1,77[a]	3,76±3,19[a]
EC100	2,98±1,33[a]	**5,17±1,62[b]**	1,85±1,71[a]
EC50	3,08±1,57[a]	**4,95±1,96[b]**	1,97±1,42[a]
L200	2,86±2,55[a]	2,54±1,51[a]	**4,6±2,86[b]**
L100	3,25±2,62[a]	3,02±1,67[a]	3,74±2,30[a]
L50	2,78±2,19[a]	2,74±1,05[a]	**4,48±1,94[b]**
EG200	2,47±2,38[a]	4,34±2,71[a]	3,19±2,92[a]
EG100	2,84±1,84[a]	3,13±2,13[a]	**4,03±2,16[b]**
EG50	2,93±2,10[a]	**5,19±2,37[b]**	1,88±2,46[a]

Note: EC200 – 200 µL eucalyptol; EC100 – 100 µL eucalyptol; EC50 – 50 µL eucalyptol; L200 – 200 µL (S)-(-)-limonene; L100 – 100 µL (S)-(-)-limonene; L50 – 50 µL (S)-(-)-limonene; EG200 – 200 µL eugenol; EG100 – 100 µL eugenol; EG50 – 50 µL eugenol.

6. Test Biodegradable Packaging

The evaluation of biodegradable films aimed at reducing plastic use and providing more sustainable packaging for stored rice was also undertaken. The commercial bioplastic Mater-Bi (biodegradable and compostable according to EN 13432 standards), alongside a traditional petrochemical plastic, BOPP (Biaxially Oriented Polypropylene) with a 30 µm transparent layer and a 70 µm transparent PE layer were evaluated. For the test, five replicates of 50 grams of rice were each infested with 50 insects and then packaged in both types of films. CO_2 levels in these samples were measured using a portable sensor. It quickly became apparent that the biodegradable Mater-Bi bags were not a viable alternative to plastic, as insects easily pierced the film, allowing them to escape. Figure 6 illustrates that CO_2 levels in the samples with biodegradable film were comparable to those of the control, due to air exchange through the insect-created holes.

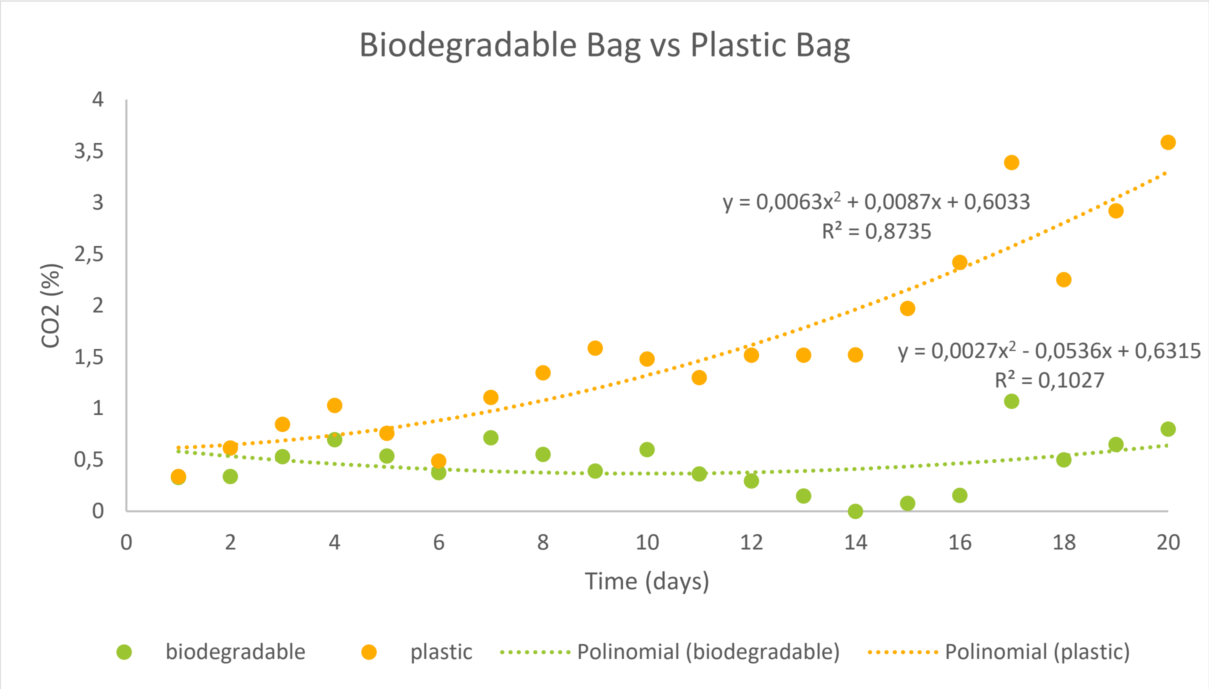

Figure 6 - Mean CO_2 values (%) throughout the insect life cycle, measured on rice packed with biodegradable versus plastic film.

In this context, as detailed in deliverable D2.3, an active packaging solution incorporating Natural Deep Eutectic Solvents with thymol was developed for testing as rice packaging (Marsin & Muhamad, 2023; Wongphan et al., 2023). Unfortunately, this membrane proved to be very brittle and fragile, rendering it impractical for handling and packaging.

7. Exploring Electromagnetic Wave Treatment

The growing interest in developing alternatives to conventional treatments has led to the exploration of electromagnetic waves, such as ultraviolet (UV) light and radiofrequency waves, for disinfecting food products. UV light, a non-ionizing portion of the electromagnetic spectrum, can be divided into three ranges: UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm). UV-C is particularly noteworthy for its well-established germicidal properties (Dittgen et al., 2021). Although higher doses or intensities of UV-C theoretically increase disinfestation efficiency, this effect may be limited in solid foods due to the insufficient penetration of UV-C rays into the food matrix (Fan et al., 2017).

In this context, collaborative trials with the iBET – Instituto de Biologia Experimental e Tecnológica are underway, where 5 replicates of 3g of previously infested rice are treated with UV LEDs at 280 nm for 15, 30, and 40 minutes (Figure 7). The goal is to compare treated and untreated samples to determine whether UV treatment inhibits insect behaviour, specifically by preventing egg hatching and inhibiting insect development. Depending on the results, further studies may explore the effectiveness of other UV intensities, such as 260 and 270 nm.

Figure 7 - Ultraviolet equipment developed by iBET – Instituto de Biologia Experimental e Tecnológica, designed for treatments using LEDs at various intensities (260, 270, and 280 nm).

These results will guide the selection of the most effective radiation wavelengths and exposure times for further validation and replication in food disinfestation efforts.

8. Conclusion

To prevent insect damage in rice, companies should prioritize the thorough cleaning of facilities, as storage infestations are nearly unavoidable. Additionally, efforts must be made to provide promising alternatives for preventing weevil infestations originating in the field.

The TRACE-RICE project conducted a series of trials to identify solutions for preventing egg hatching and controlling insect infestations in stored rice, aiming to replace the use of chemicals in the industry.

The findings suggest that these innovative solutions could effectively maintain rice quality and reduce reliance on chemical treatments.

One effective method of controlling infestations is through prevention, using techniques such as qRT-PCR and the O_2/CO_2 portable sensor to detect hidden infestations. The study selected the impregnation of rice with essential oils Limonene, Eugenol, and Eucalyptol and the application of UV radiation as solutions for further validation and replication.

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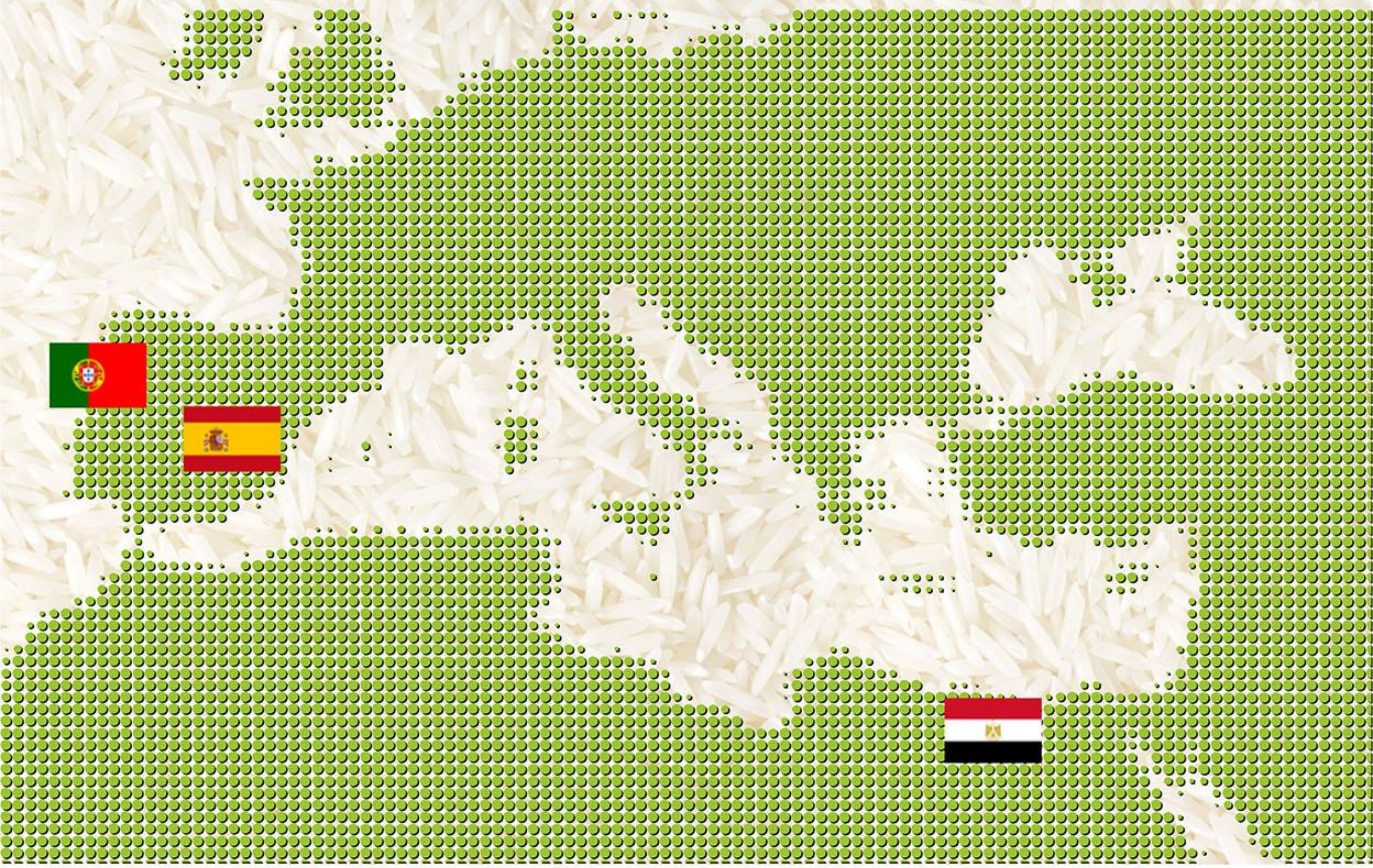

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