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Detection of hidden insect *Sitophilus oryzae* in wheat by low-field nuclear magnetic resonance

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Abstract

Insects, either adults or larvae, living inside grains are difficult to detect but can cause enormous loss of grain. Therefore, we explored the use of low-field nuclear magnetic resonance (LF-NMR) techniques to detect *Sitophilus oryzae* hidden inside wheat. Significant difference in transverse relaxation times (T_2 /ms) and the T_2 components proportion (P_2 /%) was observed between wheat and *S. oryzae* at its four different growth stages (small larvae, large larva stage, pupal stage and adult stage). The transverse relaxation signals on the infested

wheat kernels varied with *S. oryzae* developmental stages. LF-NMR image of uninfested wheat were very different than infested wheat with the hidden insects at its four growth stages. Therefore, LF-NMR, as a novel non-destructive method, could be used to detect insects hidden in grains to take necessary management against pest damage to grains during storage.

Keywords Rice weevil (*Sitophilus oryzae*); Grain storage; Transverse relaxation signal; Infested wheat; Low field magnetic resonance imaging

1. Introduction

Insect infestation in stored grain is a worldwide problem. Insect pest infestations in grain are responsible for qualitative and quantitative losses of 5 - 10% grain losses in developed countries and 30 - 40% in some developing countries (Brader et al., 2002; Kumar and Kalita, 2017) [ENREF 1](#). Insect detection is a prerequisite of Integrated Pest Management, a process often used to solve pest problems while minimizing risks to people and the environment. Many methods have been developed for the detection and monitoring insect infestations, including visual inspection, sampling & sieving, floating & cracking (Brader et al., 2002), trapping (Hagstrum et al., 1998), bioacoustic methods (Mankin et al., 2011), computer vision (Ridgway et al., 2002), near-infrared hyperspectral imaging (Kaliramesh et al., 2013; Singh et al., 2009), and soft X-rays (Karunakaran et al., 2003). Recently, some new techniques have emerged, such as microwave heating (Jian et al., 2015), zymography (Piasecka-Kwiatkowska et al., 2014), solid phase micro-extraction (Laopongsit et al., 2014), and biophotonic methods (Shi et al., 2016). However, most of these approaches cannot be commercialized due to various issues including throughput limits, unreliability, labour costs, time consuming and safety concerns. The most common used techniques are sieving samples or the probe-and-trap methods. It is important to note that exceptions to the probe-and-trap include the United States, where visual images are used in combination with probes, and Canada, where the Berlese funnel method is mandated (Sabu et al., 2011). These time-consuming techniques have low accuracy, which detect adult insects and ignore larvae growing inside the kernels. Primary pests, such as the granary weevil, the rice weevil, the maize weevil, the lesser grain borer, and the Angoumois grain moth, cause most of the damage to grains in storage and transportation. The larvae of the primary insect pests live entirely inside the kernel and are hard to detect. Therefore, a rapid, simple, and accurate method for detecting internally feeding life-stages of insects in grains is highly desired by grain industry and inspection and quarantine branch.

Low-field nuclear magnetic resonance (LF-NMR) is a sensitive and non-destructive technique, and widely used to detect water characteristics in numerous systems (Greiff et al., 2014; Hills, 2006) [ENREF 15](#). Typically it detects ingredients with high numbers of hydrogen protons after the food is processed or stored such as starch (Ritota et al., 2008), salted fish (Carneiro et al., 2016), blanching sweet corn (Shao and Li, 2012, 2013), ripening bananas (Raffo et al., 2005), and drying wheat (Ghosh et al., 2006). The difference in moisture is directly proportional to signal produced, which allows or the NMR to identify regions of higher moisture. Grain is typically stored at 12%-18% moisture, whereas the moisture content of insects about 65% (Shrestha and Baik, 2013), suggesting that NMR technology could be a promising approach to detect insects. The difference in moisture between insects and wheat kernels has been used to detect insects in the grain by electrical conductivity (Pearson and Brabec, 2007; Pearson et al., 2003) [ENREF 23](#) [ENREF 23](#). However, the sample must be milled before determination. It is a destructive method, whereas NMR require no sample destruction. Previously, only two studies using NMR were done on detecting insect infestations in grain (Chambers et al., 1984; Street, 1971). In those experiments, grain weevils (*Sitophilus granarius*) were detected at different stages of development inside of grain kernels by measuring chemical shifts using high-field NMR. Even though the technique showed promising results, research in using NMR for insect detection ceased, due to a lack of scientific expertise and high equipment costs. With the development of economical low-field NMR instrumentation, the use of low-field NMR for quality monitoring has recently become feasible in agri-food sciences. Therefore, we explore here the possibility with low-field proton NMR relaxation signal measurement

and magnetic resonance imaging (MRI) to detect hidden insect at different growth stages within wheat.

2. Material and methods

2.1. Insect sample preparation

Sitophilus oryzae was reared on whole wheat kernels in the National Engineering Laboratory of Grain Storage and Transportation, Nanjing, China. Approximately 500 adult *S. oryzae* were mixed with 1 kg of wheat (about 13.5%, w/w) in a wide-mouth glass bottle covered with a cotton cloth, and were maintained in a growth chamber at $29 \pm 1^\circ\text{C}$ and $65 \pm 2\%$ relative humidity. After 48 hours, all of the adults were removed from the bottle. *S. oryzae* at 4 different stages (small larvae, large larvae, pupae and adult as shown in Figure 1) were separated and collected from the infested wheat by a knife after 15, 20, 25 and 40 d, respectively. The samples were tested after collecting.

2.2. Infested wheat preparation

Approximately 400 adult *S. oryzae* were mixed with 20 g whole wheat for 48 h for infested wheat kernels. High population density of pests increases the probability of having infested kernels. Wheat kernels with single pest egg were collected under a microscope (Chongqing Guangdian Instrument Corporation Ltd., Chongqing, China). The infested kernels were divided into 2 g per jar, and cultured in a chamber at $29 \pm 1^\circ\text{C}$ and $65 \pm 2\%$ relative humidity. The samples were tested at 5, 10, 15, 20, 25 and 30 d. Some adults of *S. oryzae*, which emerged from the wheat, were removed before 30 d test.

2.3. Moisture content measurement

The moisture content of wheat kernels was determined using the whole-grain oven drying at 130°C for 19 h (ASABE, 2009). About 1 g of *S. oryzae* at each growth stage was dried at 70°C for 48 h to a constant weight, placed in a desiccator for 12 h, and then weighed (Singh and Sinha, 1977). Each sample was done in triplicate.

2.4. LF-NMR measurements

Transverse relaxation measurements were proceeded on NMI20 Benchtop Pulsed NMR Analyzer (Shanghai Niumag Corporation Ltd., Shanghai, China) operating at a resonance frequency for protons of 22.6 MHz. Spin-spin relaxation time (T_2) was measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence, a common and stable sequence for T_2 measurement (Carr and Purcell, 1954; Meiboom and Gill, 1958). Pests (0.2 g) or infested wheat (2 g) were placed in a 15-mm diameter glass test tube. The T_2 measurements were made with τ value (time between 90° and 180° pulses) of 100 μs . Data from 10,000 echoes were acquired as 32-scan repetitions at 32°C . The repetition time between subsequent scans was 1.5 s. Relaxation data were calculated by MultiExp Inv Analysis software (Shanghai Niumag Corporation Ltd., Shanghai, China) and the CONTIN algorithm was used for the multi-exponential fitting analysis.

2.5. LF-NMR image measurements

Magnetic resonance imaging was done on NMI20 Benchtop Pulsed NMR Analyzer. For these measurements, the following specifications were used: equipment field of view = 80×80 mm; matrix size = 192×256 ; echo time = 5.9 ms; repetition time = 160.0 ms, number of scans = 256; and slice width = 10.0 mm. Using these setting, proton density images were measured.

2.6. Statistical analysis

Statistical analyses were done with SPSS software (Version 16.0, Inc., Chicago, IL) and graphical data were generated with Origin Pro 8.5. One-way analysis of variance (ANOVA) followed by Duncan's

multiple comparisons test (at 0.05) was carried out. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Moisture content of wheat and *S. oryzae*

Figure 2 shows the moisture content of wheat and *S. oryzae* at four growth stages. From the larval stage to adult stage, the moisture content decreased from 66 to 44%, and moisture content of the wheat kept at about 13.5%. Moisture content of *S. oryzae* at every growth stage was much higher than that of the wheat kernel. In storage, insects can obtain water through three ways, consuming food, absorbing from unsaturated air, and from metabolic activities, such as oxidation of carbohydrates and fats (Arlian, 1979; Devine, 1978; Yaowaluk et al., 2008). Since the moisture content of wheat was low, the insects must rely on water obtained from unsaturated air or through metabolic activity. It is the basis to get different signals from grains and insects according to their moisture differences in subsequent experiment.

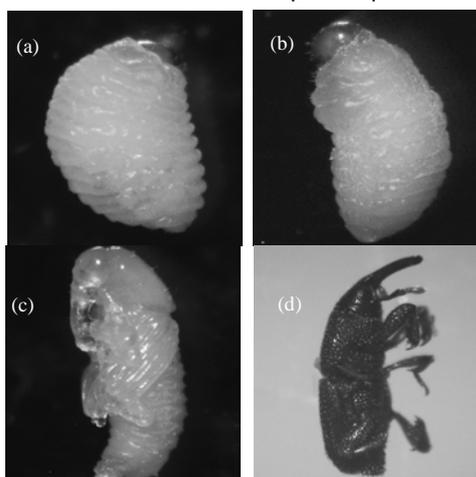


Figure 1 The life cycle of the *Sitophilus oryzae* (rice weevil): (a) small larva, (b) large pupa, (c) pupa, and (d) adult. Photographs are at about $\times 20$ magnification.

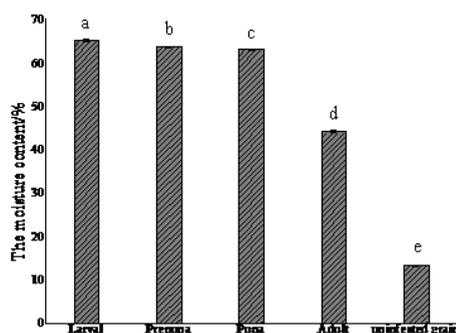


Figure 2 Moisture content of the four *Sitophilus oryzae* growth stages and unfested grain.

3.2. Transverse relaxation signal analysis for wheat and *S. oryzae*

Figure 3 shows the distribution of transverse relaxation times T_2 for *S. oryzae* adults and wheat kernels. Two peaks were overlapping for a single wheat kernel (Figure 3a), whereas two separate peaks were observed for multiple wheat kernels (Figure 3b). Two separated peaks for multiple kernels was sharper and narrower than for single kernel. In Figure 3c, 1 peak was observed on single adult, but 3 peaks were obtained on about 90 adults. The peak height was directly related to the proton content and therefore low proton content in the sample reduced the detection accuracy. Accuracy of LF-NMR can be improved by amplifying the magnetic field, increasing the sampling time or by using a larger number of samples. However, amplification of magnetic field and increase sampling times, result in increased equipment costs and longer detection time. Therefore, a certain number of samples were used in the following experiment. As shown in Figure 4, the representative continuous distribution of transverse relaxation times for wheat and *S. oryzae* at the four growth stages. Two T_2 components were detected in both samples, but with different distributions for wheat kernels and insects. The intensity of the signal per unit mass of *S. oryzae* was higher than that of unfested wheat as the higher moisture content of the insect (Figure 2). The position of the insect

peaks moved leftward as they grew up, indicating that molecular mobility in insect bodies decreases with maturity.

Table 1 shows transverse relaxation times (T_2) and percentages of water component (P_2) in four different growth stages and wheat. In wheat samples, T_{2b} was the short relaxation time at 0.76 ms that was associated with strong boundary water trapped within macromolecular structures, such as starch and protein (Bertram et al., 2001; Li et al., 2014; Shao et al., 2016a; Shao et al., 2016b). The long relaxation time was T_{22} at 96.02 ms that could be the superimposed signal from water and lipid molecules, although the lipid level was very low in wheat. For *S. oryzae*, the short relaxation time T_{21} , ranging from 1.6 to 2.8 ms, was related to the protons integrated with organized protein structures and associated with weakly bound water (Bertram et al., 2001; Li et al., 2014; Shao et al., 2016a; Shao et al., 2016b). The long relaxation time T_{22} , ranging from 37 to 58 ms, could be attributed to immobilized water or capillary water located in the intercellular space or capillaries, and was significantly lower in insects compared to wheat ($p < 0.05$). The P_{22} percentages for the insects at the 4 different insect growth stages ranged from 89.53 to 91.64%, implying that the main water component was immobilized water, irrespective of the *S. oryzae* growth stage. In contrast, the main water component in wheat was strong boundary water, accounting for about 90% water. Therefore, molecular mobility in insects when compared to that of wheat kernels was significantly different as water under low moisture conditions (i.e. in case of wheat) was less mobile. This observation was particularly useful in identifying insect infestations.

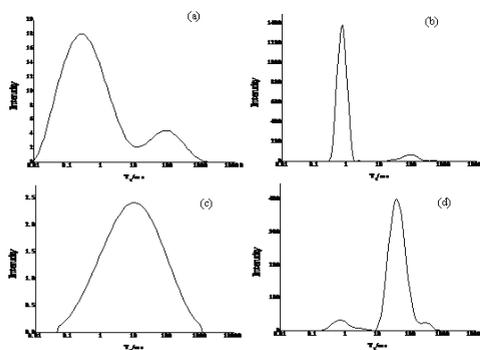


Figure 3 Typical distribution of T_2 relaxation times for *Sitophilus oryzae* adults and wheat kernels: (a) single uninfested wheat kernel; (b) multiple uninfested wheat kernels (Approx. number: 38); (c) single adult; (d) multiple adults (Approx. number: 91).

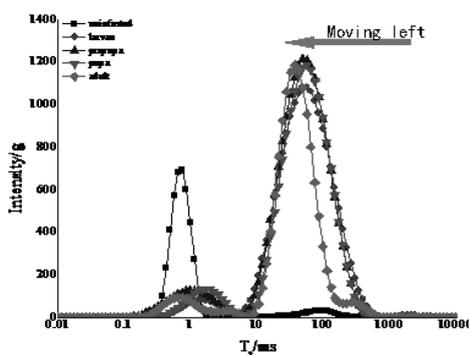


Figure 4 Typical distribution of T_2 relaxation times for *Sitophilus oryzae* at different growth stages and for uninfested grain.

Table 1. T_2 and percentages for each water component obtained by LF-NMR for *Sitophilus oryzae* at the four different growth stages and wheat

Growth stages	T_{2b} /ms	T_{21} /ms	T_{22} /ms	P_{2b} / %	P_{21} / %	P_{22} / %
Small larva	—	1.93 ± 0.47^a	57.22 ± 0.00^b	—	8.78 ± 0.58^a	90.75 ± 1.47^a
Large larva	—	1.60 ± 0.26^a	51.01 ± 2.78^b	—	7.76 ± 1.74^a	91.64 ± 1.62^a
Pupa	—	2.25 ± 0.42^{ab}	57.22 ± 0.00^b	—	7.58 ± 0.35^a	90.63 ± 1.06^a
Adult	—	2.77 ± 0.75^b	37.65 ± 0.00^c	—	9.69 ± 0.72^a	89.53 ± 0.57^a
Wheat	0.76 ± 0.00	—	96.02 ± 12.33^c	90.97 ± 0.28	—	5.01 ± 1.28^b

Note: ^{a-c} means within a column with different letters are significantly different ($P < 0.05$).

Table 2. Moisture content of the infested wheat kernels varied with *S. oryzae* growing up.

<i>Sitophilus oryzae</i> growing days (d)	Moisture content (%)
0	13.44% ± 0.12 ^a
5	13.94% ± 0.06 ^b
10	14.95 ± 0.11 ^d
15	15.19 ± 0.08 ^e
20	19.24 ± 0.08 ^f
25	20.04 ± 0.03 ^g
30	19.09 ± 0.08 ^f
Control group of wheat at 30 d	14.18 ± 0.02 ^c

Note: ^{a-h} means within a column with different letters are significantly different ($p < 0.05$).

3.3. Transverse relaxation signal analysis for infested wheat with *S. oryzae* inside

Table 2 shows that there was a significant increase in the moisture content of infested wheat when insects were developing inside the kernels. This is obvious, as a living organism inside the kernel would add moisture to its microclimate due to respiration and metabolic activity. There was a sharp rise between 15 and 20 d, probably due to metabolic water produced by the insects increased to adapt to higher growth rates. Table 3 shows the transverse relaxation time values for insects that developed inside wheat. T_{2b} ranged from 0.76 to 1.52 ms over 30 d, indicating that the water mobility in wheat changed over time. This occurred because the macromolecular structures of wheat were destroyed by *S. oryzae* to free water from the macromolecular structures and new water component was accumulated in the pest body. It can be confirmed by that the water component P_{2b} decreased after 5 d and P_{22} increased after 10 d as shown in Figure 5. These results suggest that insect infestation not only affected the total moisture content of the stored grain, but also changed the mobility characteristics inside the kernel. However, there was a decrease in T_{22} (96.02 ms to 43.77 ms) as the insects grew up. This was similar to independently detecting *S. oryzae* at each growth stages, although the correlation of this data needs further investigation in future. Especially, T_{22} with 56.75 ms at 5 d was significant from that with 96.02 ms at 0 d ($p < 0.05$), suggesting that the early growth stages of the insect could be detected by LF-NMR. The changes between water component P_{2b} and P_{22} were the opposite of each other in Figure 5. The continued increase in P_{22} from 5 to 25 d showed that the intensity of the *S. oryzae* signal increased gradually, in agreement with the total water content in the infested wheat kernels in Table 2. This is probably due to developed larger insects that contain more water in their bodies. Unexpectedly, P_{2b} increased while P_{22} decreased at 30 d. The P_2 changed in non-monotonic way whereas T_2 values were monotonously changing (seen in Table 3), varied with *S. oryzae* growing up. Only at 30 d, we found that some adult *S. oryzae* climbed out and left empty grain. All indicated that transverse relaxation signal by LF-NMR could accurately measure the whole growth of rice weevil *S. oryzae* in kernels.

Table 3. T_2 values of the infested wheat kernels varied with *S. oryzae* growing up

Infested days (d)	0	5	10	15	20	25	30
T_{2b} /ms	0.76 ± 0 ^a	0.89 ± 0.08 ^{ab}	0.92 ± 0.06 ^b	1.02 ± 0.05 ^c	1.28 ± 0.07 ^d	1.32 ± 0 ^d	1.52 ± 0 ^e
T_{22} /ms	96.02 ± 12.33 ^d	56.75 ± 9.28 ^c	59.99 ± 9.99 ^{bc}	51.27 ± 7.37 ^{abc}	52.33 ± 4.48 ^{ab}	50.84 ± 2.61 ^{ab}	43.77 ± 6.23 ^a

Note: ^{a-e} means within a row with different letters are significantly different ($p < 0.05$).

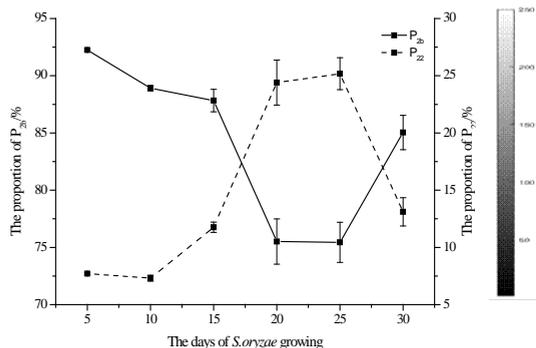


Figure 5 P_{2b} and P_{22} water component percentages of the infested wheat kernels varied with *S. oryzae* growing up.

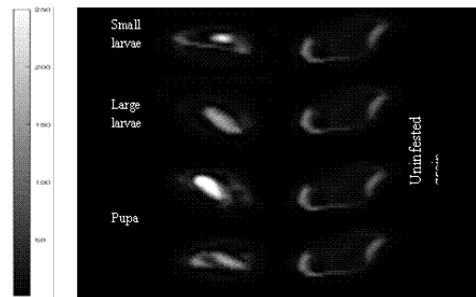


Figure 6 Low field NMR images of an infested wheat kernel at the four growth stages (left column) and for uninfested grain (right column). Sidebar stands for the grey-scale intensity of the images.

3.4. LF-NMR image analysis for infested wheat

Figure 6 shows the LF-NMR images of internally infested grain for the four growth stages and for the uninfested grain. The intensity of the signal is directly related to the sample's proton density (water or oil content); therefore, brighter zones contain larger amounts of water (Alessandra et al., 2010). Once an insect infested a wheat kernel, a bright spot appeared in the endosperm and this became enlarged as the insect grew up. This area was much brighter than the rest of the wheat kernel, suggesting that the moisture content of the insect was higher than that of wheat. This agreed with Figure 2. In addition, a bright tunnel could be seen at the adult stage because the adult had consumed the inside of the grain and was moving around freely within the grain. Therefore, LF-NMR image can visually detect insect infestation.

Conclusions

In summary, we measured and analysed LF-NMR transverse relaxation signal and image differences in wheat and wheat infested by *S. oryzae*. Our findings show that LF-NMR signal measurement or imaging could have potential to detect infested grain with hidden pests.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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IPM guidelines as fundament for sustainability in plant protection: The case for stored product protection

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Extended Abstract

According the EU Framework Directive 2009/128/EC of Sustainable Use of Pesticides (EU, 2009), member states shall implement into practice, amongst others, crop or sector-specific guidelines for integrated pest management (IPM) on a voluntary basis. In this context, article 14 demands that *“member states shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods”*. Furthermore *“Public authorities and/or organisations representing particular professional users may draw up such guidelines. Member states shall refer to those guidelines that they consider relevant and appropriate in their National Action Plans”*.

For these aims, the EU has defined eight general principles of IPM in Annex III.

These general principles comprise:

- Principle 1: Prevention and/or suppression of stored product pests where ever possible.
- Principle 2: Monitoring of pests with adequate methods and tools.
- Principle 3: Decision-making in collaboration with profesional advisors to apply appropriate plant protection measures.
- Principle 4: Practicable non-chemical measures should be preferred.
- Principle 5: Pest-specific chemical products with the lowest detectable side effects for humans, target-organisms and environment should be preferred.
- Principle 6: Where appropriate, all control measures but mainly the use of chemical products should be restricted to the minimum extent, e.g. by reducing authorized doses, and frequency of application or by partial application.
- Principle 7: Implementation of resistance strategies to maintain the efficacy of the chemical products.
- Principle 8: Documentation of all plant protection measures and evaluation of their success for future decisions.

Barzman et al. (2015) have reviewed in detail application of these eight general principles of IPM from the perspective of what their implementation means for research, advisory services and farmers. There is no doubt that practicable measures of “prevention and suppression” and “monitoring” are of utmost importance to restrict all kinds of direct interventions on a minimum and thus, to keep low risks for human health, the natural environment and groundwater. The sequence of these eight general principles can be considered as a repetitive decision-making tree where misjudgements can be adjusted in the next vegetation period(s). But, Barzman et al. (2015) conclude that there is a need for flexible, locally adapted and practical IPM strategies.

In Germany, the general principles of IPM became binding for farmers and advisors with the entry into force of the revised Plant Protection Act in February 2012. Thereby these general principles are part of the mandatory good plant protection practice. However, due to this practice and several action plans since 2003, high standards of plant protection have already been implemented in Germany for years (Hommel et al., 2013).