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Project leader:	Dr S Penfield
Report:	Report, 2020
Previous report:	None
Key staff:	Chuck Wang, University of Exeter
Location of project:	John Innes Centre, Norwich
Date project commenced:	01/04/2015

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

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SCIENCE SECTION

Introduction

The key novel biology of this project was the advances showing that changes in seed vigour are frequently accompanied by changes in seed permeability. Seed permeability is also important for seed technology, and in particular applied chemicals in seed coats are not easily taken up into seeds. Our idea was to use prior knowledge and transcriptomic analysis of temperature responses in seeds to develop a target list for the *Brassica oleracea* TILLING population at JIC. We would identify knockouts in the target list and analyse their effect on seed vigour and seed permeability. However, in order to monitor the uptake of chemicals into seeds we realised that we would need to develop new techniques, because the ability of a compound to permeate a seed coat depends greatly upon its chemical-physical properties. Therefore techniques that rely upon attachment of tracers (fluorophores and dyes) to visualise the distribution of compounds across the seed coat give a false result since they modify the transport kinetics of the compound under investigation. For this reason we have developed a label-free method for analysing uptake of AIs from seed imbibition media based on Stimulated Raman Scattering (SRS) microscopy. SRS is an optical microscopy that provides label-free quantitative imaging based on vibrational spectroscopy with sub-cellular spatial resolution. SRS has several key features that are ideal for imaging the entry of agrochemicals into seeds. (i) The near-infrared excitation permits imaging through intact seed coats. (ii) It allows tracer-free mapping of small molecules such as agrochemicals, agrochemical formulations, water and endogenous seed coat polymers with high spatio-temporal resolution and no photobleaching. (iii) The SRS can be isolated from the background from heavily pigmented plant tissues. SRS has the potential to enable radically improved design and environmental profiles of existing and future seed-applied Active Ingredients by matching AIs to seed coats with tailored permeability profiles and to improve the environmental profiles of existing Active Ingredients.

Materials and methods

Objective 1: Reverse genetics to identify *B. oleracea* strains with environmentally resilient high vigour using TILLING

We had a short list of genes affecting seed coat development in Arabidopsis for TILLING in *Brassica oleracea*. These affected seed coat suberin (GPAT5), seed coat tannin (DFR), RGL2 which affects seed coat developmental regulation by gibberellin, and AP2 which is a master regulator of seed development. To this we added MFT and tilled for mutants in each

gene (see objective 4). We obtained a large number of mutants for each gene (Table 1) which we set about back-crossing and generating homozygous lines. For most genes because we obtained up to 20 mutants we had to select the most promising mutants to focus on. This we did by focussing on mutations which led to a premature stop codon, or to an amino acid substitution where the new amino acid had different chemical properties. This led to a large number of plants to screen by developing molecular markers for each allele and using these to identify homozygous plants which could then be backcrossed to begin to remove deleterious mutations in the background. This has led to a large project growing substantial numbers of plants that is beginning to bear fruit

Gene	Name	Plant number	Generation	Genotyping result
wild type	DH1012	5		
BoAP2_C1	AP2_0665A_1	5		All Hom
BoAP2_C1	AP2_M_0697B_4*P_DH1012	5	F1	All Het
BoAP2_C7	AP2_0048B_3	5		All Hom
BoAP2_C7	AP2_M_0048B*P_DH1012	5	F1	All Het
BoAP2_C7	AP2_P_0048B_8*M_DH1012	10	F1	9 Het, 1 WT
BoDFR_C9	DFR_0750A_8	5		All Hom
BoDFR_C9	DFR_M_0750A_10*P_DH1012	5	F1	All Het
BoDFR_C9	DFR_P_0750A_7*M_DH1012	5	F1	All Het
BoGPAT_C5	GPAT5_0348A_2	5		All Hom
BoGPAT_C5	GPAT5_M_0348A_6*P_DH1012	5	F1	All Het
BoGPAT_C5	GPAT5_P_0348A_6*M_DH1012	5	F1	All Het
BoMFT_C5	MFT_C5_0439C_2	5		All Hom
BoMFT_C5	MFT_C5_M_0439C_1*P_DH1012	3	F1	All Het
BoMFT_C5	MFT_C5_P_0439C_1*M_DH1012	5	F1	All Het
BoMFT_C8	MFT_0762A_11	3		2 Hom, 1 Het
BoMFT_C8	MFT_P_0762A_11*M_DH1012	5	F1	All Het
BoMFT_C8	MFT_P_0762A_32*M_DH1012	3	F1	All Het
BoRGL2_C5	RGL2_0486B_1	5		All Hom
BoRGL2_C5	RGL2_M_0092B_9*P_DH1012	5	F1	All Het
BoRGL2_C5	RGL2_P_0092B_2*M_DH1012	5	F1	2 Het, 3 WT

Table 1. Summary of *Brassica oleracea* mutants obtained via TILLING

We also set about making double mutants where there are two copies of the gene in *B. oleracea*. These double mutants we expect to have ready in June 2019. Of these mutants, the *RGL2* gene is single copy and thus we expected that the mutants have a strong seed phenotype. Preliminary germination testing has identified that the mutants have higher germination speed under some conditions, but also that the germination is inhibited by osmotic stress (Figure 1). This shows that we can delete genes using the TILLING population and recover seed phenotypes, although we might find that this is not suitable for use by the seed industry.

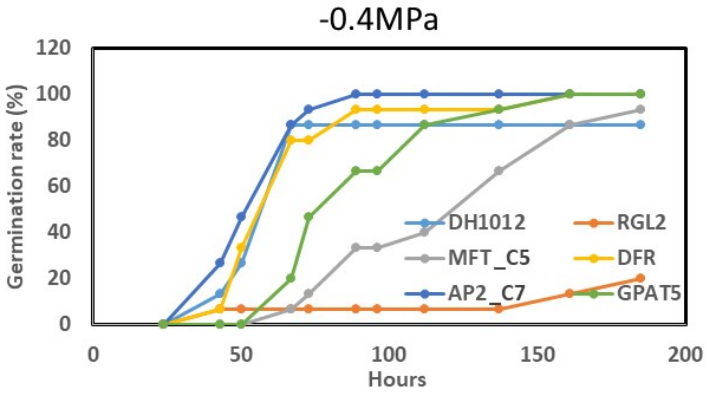


Figure 1. Germination test of single mutants under osmotic stress given by PEG solution (July 2018). Note the poor germination of *rgl2* mutant seed (orange line; germination is normal under controlled conditions).

Objective 2: Unbiased forward genetic screen to identify novel B. oleracea alleles with environmentally robust seed vigour

We conducted a forward screen of the Brassica oleracea TILLING population based on seed colour. We obtained a number of putative mutants, of which none appeared to have a strong vigour effect. Because of the success of objective 1 and the large number of plants, we decided to focus on the mutants obtained from TILLING for further analysis.

Objective 3: Field trials for seed vigour analysis

Objective 3 is slightly delayed because we are still producing homozygous seeds in the number we need for Syngenta to set at different sites. Currently Syngenta are producing lines for us at a field trial site in Kenya and these will be phenotyped for seed vigour at Syngenta and at JIC in the case of *rgl2* mutants. For the others we need to make double mutants because there are two copies of each gene in Brassica oleracea. Only after we have done this will seeds be passed to Syngenta for vigour testing.

Objective 4: To develop a mechanistic description of environmental effects on seed coat development and seed vigour in B. oleracea

A key part of the project was addressing the question of unpredictable seed maturation conditions at field sites used by seed companies, which is known to lead to variation between lots in seed quality, by affecting the properties of the seed tissues surrounding the embryo. We developed an experimental system whereby two temperatures, 16°C and 26°C, were used to produce seeds of low and high vigour respectively. By swapping seeds between the two temperatures at different points of seed development, we identified a critical

developmental window which we referred to as mid-maturation, where temperature has the largest impact on seed physiology (Figure 2).

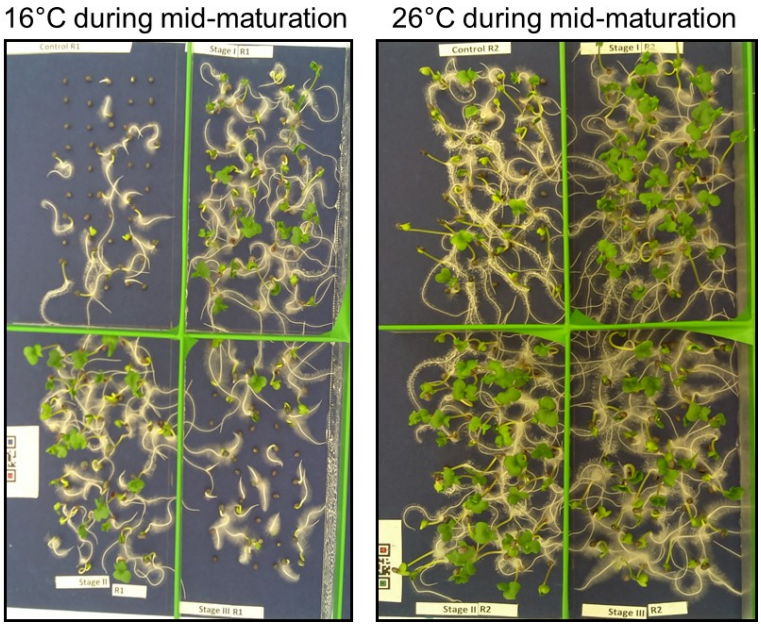


Figure 2. Seed vigour of *Brassica oleracea* seed lots produced by varying the temperature at different points during seed development and maturation. In general lots which experienced high temperature at the mid-maturation stage (right) had much stronger vigour than those which received the lower temperature (left). This lead us to conclude that temperature during mid-maturation was the key driver of vigour variation.

Furthermore, we could show that the seed tissues surrounding the embryo were important in these vigour differences because removing them at the beginning on seed imbibition substantially reduced the impact of lower temperatures on seed germination speed (data not shown). We then designed a transcriptomic experiment where we grew plants at 16°C and switched them to 26°C, harvesting embryo and endosperm/ seed coat material at 8 timepoints post transfer. The idea was to identify specific processes occurring in either the seed coat or endosperm that mediate the effect of temperature on seed quality. This lead to the discovery of an endosperm-expressed gene expression module that was subsequently highly expressed in the low temperature seeds, but whose gene expression was strongly reduced in seeds transferred to high temperatures (Figure 1). Key among these was two genes encoding the Brassica orthologues of *MOTHER OF FT AND TFL1* (*MFT*), and genes previously shown to regulate *MFT* EXPRESSION in Arabidopsis such as *SPATULA* (*SPT*). We added the *MFT* to our target gene list for TILLING. There are two copies in *Brassica oleracea*, so this adds two further targets. In addition, it is likely that we will have to make a double mutant to see a germination phenotype.

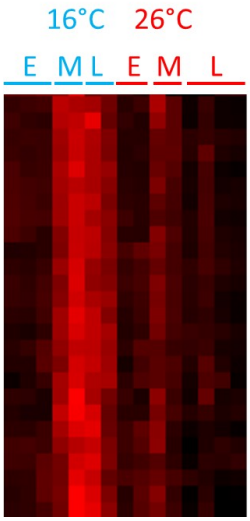


Figure 2. The expression of a temperature-regulated gene expression module in *Brassica oleracea* seeds. Gene expression is shown as a heat map with brighter red indicated higher expression. Genes with known roles in seed germination in other species are highlighted. E, M, L: early, middle and late seed maturation growth stage.

Further work in the final months of the project will be aimed at understanding which of the factors in this module alongside *MFT* might be responsible for the temperature signalling effects. This will be achieved using transient assays to see if any of the transcriptional regulators in the group can affect *MFT* expression.

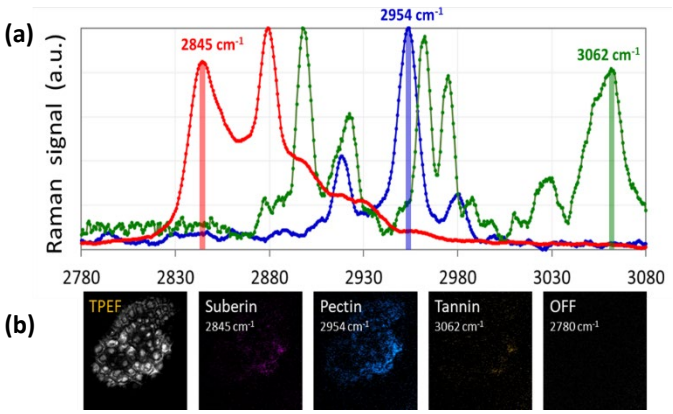
Objective 5: To develop novel quantitative microscopic tools for the non-invasive in situ analysis of environmentally regulated seed coat polymers

In-situ 3D label-free imaging of seed coat composition.

In this work package we aimed to develop a novel seed imaging technique based on SRS that provides in-situ microscopic mapping of the spatial distribution of biopolymers (i.e. cellulose, tannins and suberins) in the seed coat.

Raman analysis of the seed coat to identify spectroscopic signatures of the key seed coat polymers. In order to apply SRS to image spatial distribution of biopolymers in the seed coat it was first necessary to explore the spectral signatures of the polymers of interest. Raman analysis of purified samples of key seed coat components was performed to acquire

Figure 4



reference spectra of the key seed coat polymers in order to perform SRS imaging. This dataset formed the basis to generate label-free contrast from each component. Figure 4(a) shows example Raman spectra of the key structural components of the brassica seed coat: pectin (blue), tannin (green), and suberin (red). The peaks highlighted at 2845 cm^{-1}(suberin) 2954 cm^{-1}(pectin), and 3062 cm^{-1} (tannin) were found to provide selective SRS image, with example SRS images highlighting the spatial arrangement of each component shown in 4(b).

Spectroscopic SRS imaging to produce quantitative images of the polymeric composition of brassica seed coat in 3D in intact seeds. At the time of application we had preliminary data showing that this objective was feasible in Arabidopsis seeds. However, the thicker and heavily pigmented seed coat found in the larger Brassica seeds presented a major challenge. A significant amount of technical development was required in order to successfully image at sufficient depth into Brassica. This work involved the development of an epi-detection module to detect the signal back-scattered by the seed coat so that imaging could be performed in intact seeds; custom built electronics to isolate and amplify the weak backscattered signal to a level where we would detect the seed coat polymers; and exploration of optimal excitation wavelengths to enhance the penetration into the seed.

Compare the composition of wild type seeds set under different seed production environments, and in the novel alleles identified in Objective 1. Building from the work in the previous objective, we employed the new detection scheme to great effect to obtained 3-dimensional visualisations of the spatial distribution of seed coat polymers in Brassica seeds. Figure 5 shows example data comparing the 3D microscopic biopolymer content of the wild type seed coat against two different mutants. The upper row of images shows the (invariant) two photon excited fluorescence (TPEF) signal, highlighting the overall morphological structure. The lower row of images shows a colour merge of SRS images showing suberin (red), tannin (green) and pectin (blue). The wild type seed (Ler) shows an approximate equal contribution of each component, where the mutants deficient in tannin (tt4-1) and suberin (awe1) show a the corresponding deficiency in green and blue respectively.

Figure 5

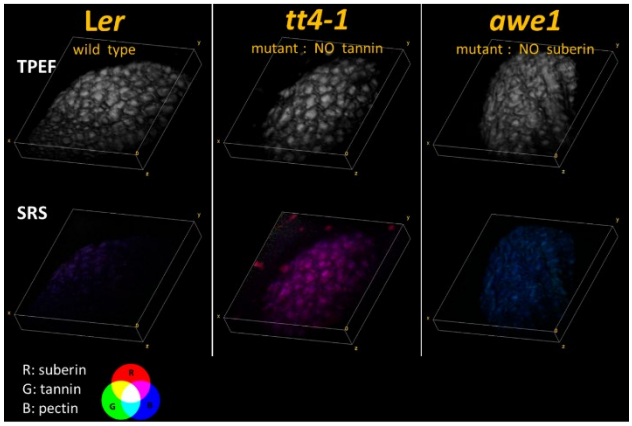
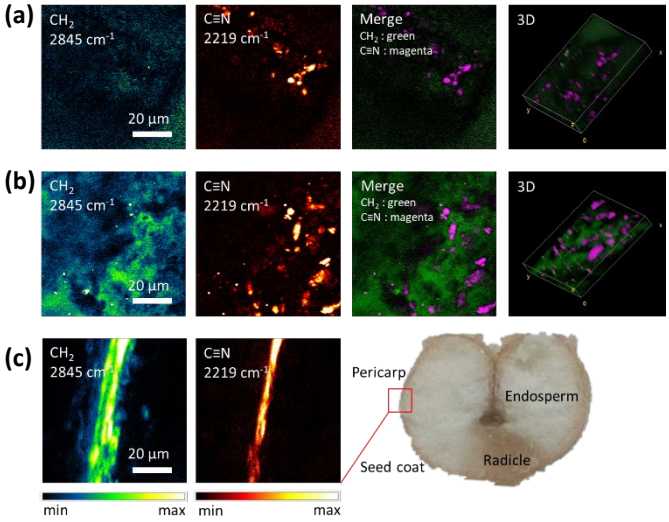


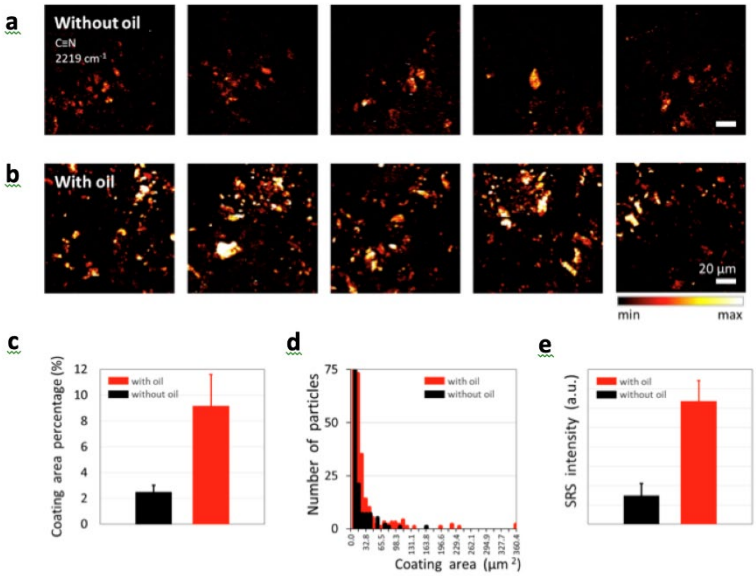
Image the spatial distribution of exogenous biopolymers and AIs in seeds treated with commercial coatings to provide novel visualisation of the thickness, uniformity, and phase-separation of biopolymer coatings and AIs at the microscopic level. Using the methods developed earlier, we demonstrated that SRS provides *in situ* quantitative analysis of the spatial distribution of exogenous biopolymers and AIs in seeds treated with commercial coatings to provide novel visualisation of the thickness, uniformity, and phase-separation of biopolymer coatings and AIs at the microscopic level. Figure 6 shows an example were we used fludioxonil, a non-systemic fungicide, to highlight that SRS can compare the distribution compounds applied to the seed coat with and without co-formulants. SRS imaging of CH_2 (2845 cm^{-1}) and C≡N (2219 cm^{-1}) on the surface of intact seeds as shown in (a) fludioxonil without and (b) fludioxonil with a formulation additive. (c) Shows images acquired from a seed cross-section to demonstrate the surface coverage.

Figure 6



Moreover, we were able to extract quantitative data on the effect that co-formulants have on the microscopic distribution of AI on the seed surface. Figure 7 compares the distribution of fludioxonil on the surface of seeds coated with and without an oil-based additive that was believe to enhance the coating. (a) and (b) compare the coating performance of fludioxonil mixed without the additive, while (c) provides a quantitative analysis of coating area percentage, (d) the number of fludioxonil particles, and (e) the overall quantity of AI per unit area.

Figure 7



Objective 6: To develop novel quantitative microscopic tools for the non-invasive in situ analysis

Monitoring active ingredient diffusion in real-time.

In this work package we built upon the spectroscopic analysis developed in workpackage 5 to develop techniques that provide time-resolved chemical mapping of the seed coat as function of depth to explore, for the first time, the diffusion of unlabelled compounds into the seed coat with microscopic resolution.

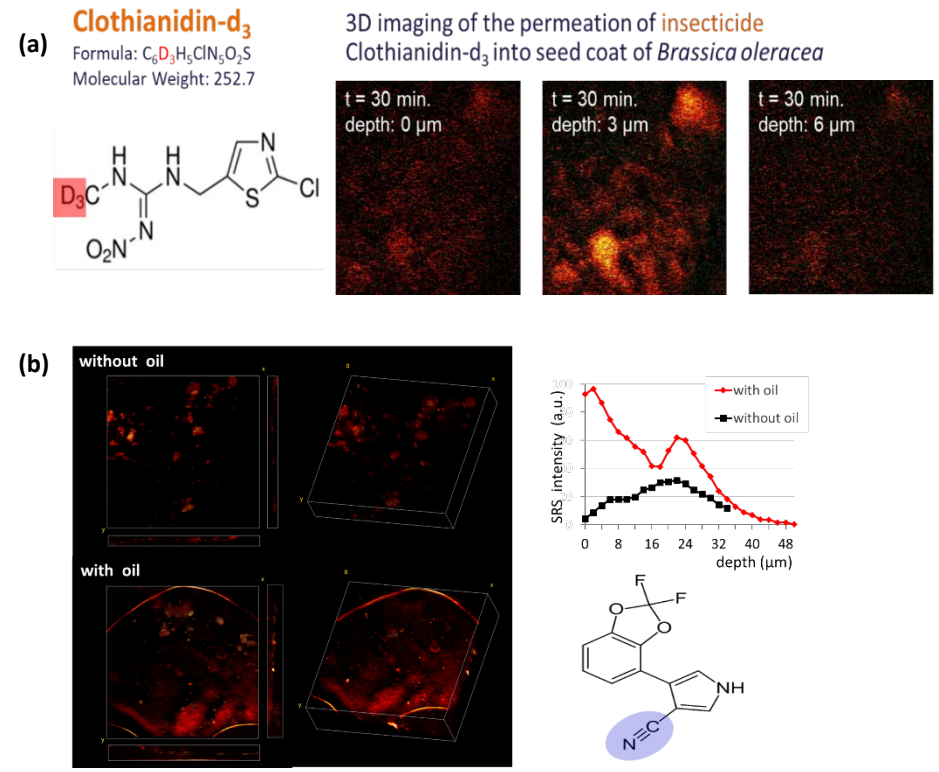
Identify a range of low molecular weight AIs, solvents, emulsifiers, excipients and agrochemical polymer coatings with chemical motifs with Raman active modes that provide contrast against the seed coat. Working with our industry partner (Syngenta), we explored a wide range of chemistries commonly used in commercially applied seed coatings to identify chemical motifs with Raman bands that could be exploited for SRS contrast. Due to their strong and pronounced peaks in biologically silent regions, cyano and carbon-fluorine groups were found to be good candidates commonly occurring in commercial AIs. For AIs without these motifs we found that deuterated versions of AIs, available in compound libraries for

NMR analysis, could be used to provide chemical contrast without significant chemical perturbation of the chemical-physical properties. For example, the deuterated version of the insecticide Clothianidin (Clothianidin-d3) provides a unique Raman band at 2083 cm^{-1} that could be used to image its distribution in the seed coat. Water being the most relevant solvent, and with an MW of only 18 cannot be labelled without drastically modifying its diffusion coefficient. For this reason, we explored the use of the Raman signal from the O-D stretch of deuterated water to track the uptake of this extremely important solvent molecule. With a MW of 20, D_2O provides a close approximation to water and has a peak in a biologically silent spectral region. We also explored the Raman signitures of commercial emulsifying agents and excipients. Oil-based agents were found to be relevant as additives to enhance the efficacy of commercial seed coatings and exhibit extremely strong Raman bands at the CH_2 stretching that could be used to map there distribution with SRS.

Time-resolved chemical mapping of the seed coat as function of depth to visualise the uptake of unlabelled molecules with various physical-chemical properties. Building upon the spectroscopic analysis developed in objective 5, we developed techniques to provide real-time chemical mapping of the seed coat as function of depth. The sensitivity enhancement achieved in **objective 5** allowed us to rapidly acquire 3D hyperspectral (X,Y,Z, frequency) SRS data sets as a function of time. We were able to visualise the route of uptake of unlabelled molecules into intact seeds and explored the penetration of compounds with a range of physical-chemical properties with unique Raman bands identified in **objective 5**.

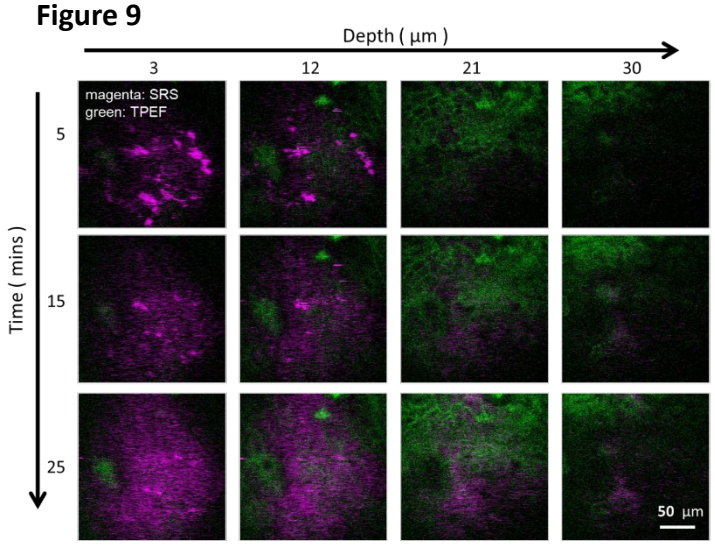
Using the chemical motifs identified in previous objective we were able to image the distribution applied AI seed coating and where relevant their diffusion into the seed coat. Figure 8a shows example data of 3D imaging of the permeation of the insecticide (Clothianidin-d3) into seed coat of a Brassica oleracea seed. Diffusion was observed to take place over 30 minutes up to a depth of 10 microns. Moreover, we were able to visualize the effect of co-formulants on the uptake of AIs. Figure 8b compares data of fludioxonil, a non-systemic fungicide with a cyano bond. As well as increasing the thickness of the applied seed coating oil-based co-formulations were observed to increase the penetration into the seed by a factor of 3 up to 40 microns. The initial increased AI at the surface is due to the thicker coating resulting from the oil-based additive.

Figure 8



We used the SRS signal from D_2O to great effect to acquire real-time image data showing the movement of water into the seed coat of intact Brassica seeds. We have acquired a significant number of time-lapse data sets of water diffusion into different seed types as a function of depth. Figure 9 shows an example data set demonstrating the level of detail that can be seen as a function of depth and time. The seed coat is shown in green and the SRS signal from deuterated water in magenta. This is the first time that anyone has been able to visualise the movement of water into the seed

Figure 9



coat with such high spatial and temporal resolution. We are currently analysing the data with biophysical models to make quantitative comparisons between different seed mutants.

Results

Outcomes so far:

1. Novel Brassica oleracea alleles that may affect seed vigour. Markers to track these alleles through breeding populations
2. Development and application of a novel label-free, real-time imaging technique for visualising the distribution of compounds in the seed coat at the microscopic level. The ability to visualise the movement of unlabelled compounds into the seed coat on this scale represents a major advance in analytical capability for both plant biologists and agrochemical scientists.
3. New understanding of the mechanism by which temperature during seed set affects seed vigour in Brassicas, by regulating MFT expression.

Knowledge and Technology Transfer

Publications so far:

Wang et al (2018). In situ chemically specific mapping of agrochemical seed coatings using stimulated Raman scattering microscopy.

Other activities:

Visit to Syngenta Enkhuizen, May 2016

Meet with Syngenta seeds, July 2018.

Presentation to JIC plant breeders day June 2016.

Visit to Produce World, September 2016.

Presentation to International seed testing meeting, June 2014.

Presentation at 3 HAPI meetings.

Presentation at Plant dormancy conference, Kyoto October 2018.

Article in AHDB grower magazine, September 2017.

Photonics West, USA 2017

European Conference in Non-linear Optical Spectroscopy (ECONOS) 2018

Analytica 2016, Germany

Microscopy Society of Ireland 40th Annual Meeting 2016, Ireland

Nordic Microscopy Society SCANDEM 2016, Norway

Imaging and Advanced Spectroscopic Methods Workshop, African Laser Centre, South Africa 2016

