HR-MAS NMR-based metabolomic approach to study the effect of fungicidal stress on wheat seed germination

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The plant metabolome is sensitive to variations in the growth environment, as evidenced by metabolomic profiling. Changes in the levels of specific metabolites can be quantified via high-resolution magic angle spinning (HR-MAS) nuclear magnetic resonance (NMR) spectroscopy and can help identify key metabolic pathways related to environmental stress. We study here the metabolic response of wheat seeds to fungicidal stress during germination. A delay in germination and a decrease in percentage germination were observed for fungicide-treated seeds. Our work validates the use of HR-MAS NMR spectroscopy as a novel method to gain quantitative information about the influences of micro-stresses on nutritional backgrounds in plants.

Keywords: Fungicidal stress, germination cycle, HR-MAS NMR spectroscopy, metabolite fingerprinting.

HIGH-RESOLUTION nuclear magnetic resonance (NMR) spectroscopy is a powerful and non-destructive approach to quantify plant metabolites and to understand fundamental biotic and abiotic plant phenomena[1–5]. Multivariate statistical analysis of NMR metabolite data has led to the quantification of metabolic variation in a wide variety of plants and bioorganisms[6–9]. High resolution magic angle spinning (HR-MAS) NMR combines high-resolution solution NMR techniques with rapid sample rotation at the magic angle, in order to reduce line broadening due to anisotropic interactions[10]. HR-MAS NMR has a number of unique advantages over other techniques such as GC-MS and LC-MS, as it is a non-destructive, non-biased and quantifiable technique requiring no chemical derivatization, and provides easy identification of novel compounds such as sugars and ketones that are less amenable to GC-MS or LC-MS analysis[11–14].

The present work focuses on applying HR-MAS NMR methods to study the effects of fungicide treatment on the metabolite response of germinating wheat seeds. The application of metabolomics for the discovery of the bioaction of pesticides (including herbicides, fungicides and insecticides) has been recently reviewed[15]. It has also been shown that phytotoxins have an adverse effect on germination and growth parameters[16]. Hence although seed treatments have important benefits, they also pose a risk of phytotoxicity. Since metabolic responses to environmental stress regulate plant growth, it is important to study the phytotoxic effects of fungicides. In this study, the effects on wheat seed germination of two different kinds of fungicides were quantified: a non-systemic fungicide thiram (N,N-dimethylcarbamodithioate) and a systemic fungicide bavistin (methyl-N-1H-benzimidazol-2-yl-carbamate). Both thiram and bavistin have been widely used for the protection of wheat and other seeds and for treating seed-borne pathogens[17,18]. Changes in plant metabolism due to fungicidal stress are reflected in the NMR metabolite spectra and were used to quantify the effect (at different time points after imbibition) of adding fungicides to the growth environment. Significant changes in spectra occurred during different stages of the germination cycle as evidenced from the time-series plots showing the changes in the lipid, sugar and betaine metabolism over different imbibition times. Significant metabolites identified through supervised partial-least-squares discriminant (PLS-DA) analysis were used as markers for the stress experienced due to fungicide treatment and to study time-dependent metabolic fluctuations under such stresses during the germination cycle. The results underscore the relationship between changes in metabolite concentrations in the HR-MAS NMR spectra and the underlying biological processes, i.e. the effect of fungicide phytotoxicity on the metabolic pathways involved in seed germination. This study hence points the way toward using HR-MAS NMR methods to broadly identify the impact of fungicides on metabolic pathways during germination.

Surface sterilization of wheat (*Triticum aestivum*) seeds was done by soaking the seeds in 1% NaOCl solution for 5 min. The seeds were then rinsed thrice in distilled water under a laminar hood. For the fungicidal growth environment (in the absence of fungal infection), seeds were treated with two types of fungicides: bavistin and thiram, each having a concentration of 6 mM, made by weighing an appropriate amount of fungicide and dissolving in distilled water to make a paste. The seeds were then surface coated with the fungicidal paste and allowed to dry under shade. For a control environment, the seeds were only surface sterilized and no fungicidal treatment was done.

Seeds which were fungicide-treated and the untreated seeds were allowed to germinate. For germination experiments, four replicates of 100 seeds each were used for each type of treatment (untreated, systemic and non-systemic fungicide). Seeds from all three treatments were separately placed in 8.5 cm glass petri dishes on a layer of filter paper (Whatman No. 4) made wet by adding distilled water in required amounts. Seed germination was recorded after every 12 h of imbibition, till day 5. A seed was considered germinated when a radicle (about 2 mm in length) emerged. Seeds from each treatment were scored for radicle emergence after every 12 h of imbibition.

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The cumulative germination percentage was calculated for each seed treatment.

Both fungicide treated and untreated seeds were separately placed in 8.5 cm glass petri dishes containing wet filter papers. The seeds were incubated and germinated at 25°C under a 12 h light (8 : 30 am to 8 : 30 pm)/12 h dark (8 : 30 pm to 8 : 30 am) cycle in a growth chamber (Vista Bio Cell Pvt Ltd, India). Seeds were observed every day till the fourth day from imbibition (96th hour). Ten replicates of each type (systemic, non-systemic and control) were observed after every 12 h from the start of the experiment. Each replicate accounted for an average of ten seeds (a total of 100 seeds for each type of seed treatment), which were crushed using a pestle and mortar and mixed thoroughly, and the sample prepared by adding 10 l of 0.2 M phosphate buffer (pH 7) in D_2O prior to NMR experiments. The sample was packed into a 4 mm zirconium oxide HR-MAS rotor with a 12 µl spherical insert and a Kel-F cap.

HR-MAS NMR spectra were recorded on a Bruker Biospin 600 Avance-III spectrometer operating at a ^1H frequency of 600.13 MHz at 298 K and equipped with a high-resolution MAS probe. Samples were spun at 2 kHz at the magic angle. Three different kinds of 1D ^1H NMR spectra were acquired: (i) 1D spectra with water presaturation achieved by cw irradiation applied during the relaxation delay to suppress the residual HOD signal; (ii) 1D spectra with water suppression using the NOESY presat sequence, and (iii) a water suppressed Car-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence optimized for a total time of 0.3 ms. The proton 1D spectra were collected with a 90° pulse width of 5.75 sec, a relaxation delay of 1 sec, 16 scans, 64 K data points and a spectral width of 12335.53 Hz. The spectra were processed with a line broadening factor of 0.3 Hz prior to Fourier transformation with automatic phasing and polynomial baseline correction. Wheat seed spectra were referenced (internally) to the betaine resonance at 3.27 ppm. For resonance assignment and metabolite identification, a set of two-dimensional NMR spectra were recorded, including ^1H–^1H correlation spectroscopy (COSY), ^1H–^1H total correlation spectroscopy (TOCSY), ^1H–^1H nuclear Overhauser effect spectroscopy (NOESY) and ^1H–^{13}C heteronuclear single quantum coherence spectroscopy (HSQC). 2D ^1H–^{13}C HSQC spectra were obtained with a spectral width of 12 and 280 ppm in the proton and carbon dimensions respectively, 1 K data points, four scans, 256 t_1 increments and a recycle delay of 1.5 sec. The COSY and TOCSY spectra were acquired with a spectral width of 12 ppm in both dimensions, 2 K data points, eight scans, 128 t_1 increments and a TOCSY mixing time of 60 ms. The 2D NOESY spectra were acquired with a spectral width of 20 ppm, 2K data points, 256 t_1 increments, 32 scans and a mixing time of 300 ms.

Metabolites were identified based on the NMR chemical shift values obtained from 1D and 2D NMR experiments. Metabolite fingerprinting for the wheat seed spectra was done using a semi-automatic assignment method, Metabohunter[19]. The results were checked with standard NMR metabolite data deposited in databases such as the Biological Magnetic Resonance Data Bank (BMRB) (http://www.bmrb.wisc.edu) and the Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu).

For the statistical study, 10 replicate samples for each type of seed treatment (bavistin treatment, thiram treatment and no treatment) were used over different imbibition times during germination cycle (day 0 corresponding to the ungerminated seed). The NMR variables used for the multivariate statistical analysis were generated by binning each NMR spectrum in the 0–12 ppm range with a bin width of 0.04 ppm using the software package MNova from the MestRe-C Lab, Spain (http://www.mestrec.com/). The spectral region 4.7–5.1 ppm was excluded from the analysis to correct for incomplete water suppression and cross-saturation effects. Data were normalized by the constant sum method. Principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed with the Pareto method for scaling, using the Metaboanalyst software (http://www.metaboanalyst.ca)[20].

The results of the germination experiment showed variation in germination between different fungicide treatments and untreated controls (Figure 1). The impact of fungicides on seed germination was observed in the form of a delay in seed germination and reduction in germination percentage being more pronounced in thiram-treated seeds compared to bavistin-treated seeds (Figure S1, Supplementary Material online). Germination of untreated controls could be observed by the second day, which reached to 100% germination by the third day. Only 9% germination was observed in bavistin-treated

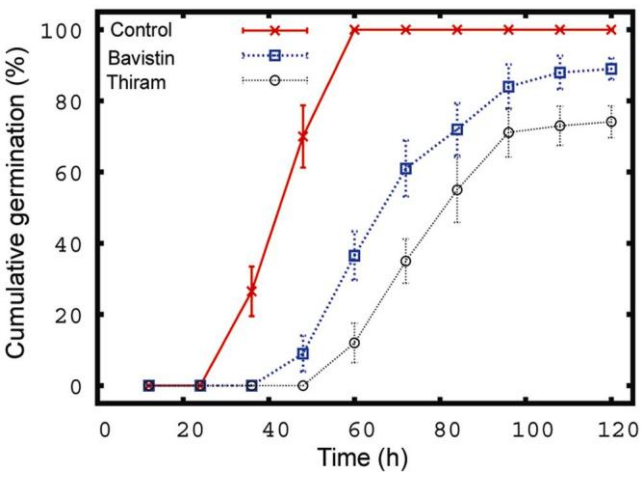

Figure 1. Cumulative percentage of germination over five days (120 h) for bavistin-treated wheat seeds, thiram-treated wheat seeds and untreated control seeds. Each point is an average of four replicates (of 100 seeds), with bars indicating standard error.

seeds on the second day, which reached 89.5% germination by the fifth day. In the case of thiram-treated seeds, germination could be observed only by the third day, which reached a maximum of 74% by the fifth day.

A typical 1D ^{1}H HR-MAS NMR spectrum of a wheat seed recorded at 600 MHz is shown in Figure 2, with the chemical shift resonances corresponding to identified metabolites listed in Table 1. 1D ^{1}H solution NMR spectrum of fungicides bavistin and thiram were also recorded at 600 Mhz (Figure S2, Supplementary Material online). The CPMG pulse sequence for the HR-MAS spectra gave the best S/N ratio of the 1D NMR sequences. The metabolite identification was achieved by analysis of the 1D and full set of 2D data (Figures S3 and S4, see Supplementary Material online). The metabolic profile of the wheat seeds contains a range of amino acids, sugars and lipids. The amino acid peaks show up clearly in the solution state spectrum (Figure S5, see Supplementary Material online) while the lipid peaks show up better in the HR-MAS spectrum (these peaks are missing in the solution state spectrum).

1D HR-MAS NMR data were statistically analysed to determine the extent to which the selected variables (metabolites) are able to distinguish between the different types of seeds (fungicide-treated and control). Initially PCA was applied to the binned data of fungicide and control seeds (Figures S6 and S7, see Supplementary Material online). We then used the supervised method of PLS-DA to further enhance the separations and identify the metabolites associated with the effects of fungicidal treatment. We obtained better grouping of clusters using PLS-DA compared to the PCA model. Figure 3 *a–d* shows plots of the PLS-DA component scores, over a time period of four days from the start of imbibition, for the most important components (PLS-component 1 versus PLS-component 2) for wheat seeds which are treated differentially (with and without fungicide). The cluster analysis of NMR data from the score plot shows a clear distinction between the fungicide-treated seeds and control seeds. Figure 3 *a* shows a clear separation between control seeds and fungicide-treated seeds on day 1 itself, even though there was less distinction visibly. All seeds are compared to day 0 seeds to show how metabolic changes occur as germination progresses from day 0 to day 4. Spectra of both bavistin and thiram-treated seeds resemble the ungerminated dry seeds of day 0. As the days progress (Figure 3 *b–d*), the bavistin-treated seeds show less separation from control seeds. Over time, all seed types germinate and undergo metabolic changes compared to ungerminated dry seeds of day 0. However, germination in fungicide-treated seeds shows a clear retardation compared to untreated seeds that show normal germination. The PLS-DA analysis showed distinct separation (R^2Y) and good predictability (Q^2Y) for all time points (day 1: $R^2Y = 0.90$ and $Q^2Y = 0.78$, day 2: $R^2Y = 0.84$ and $Q^2Y = 0.79$, day 3: $R^2Y = 0.92$ and $Q^2Y = 0.84$, day 4: $R^2Y = 0.88$ and $Q^2Y = 0.84$). Further validation of the PLS-DA model was performed using the permutation test on the best model. The *P* value obtained

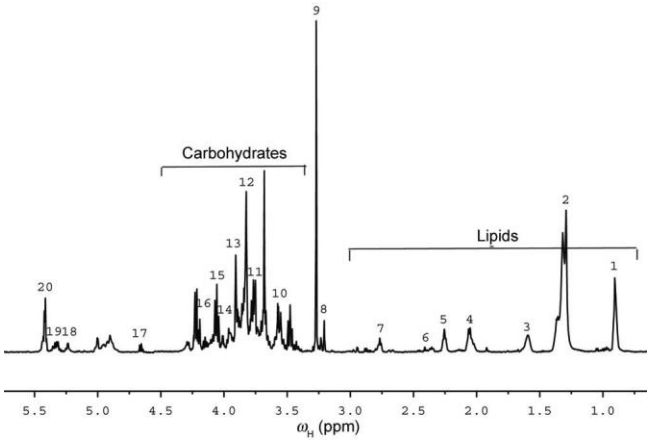

Figure 2. Wheat 1D ^{1}H HR-MAS NMR spectrum recorded at 600 MHz showing specific resonances of sugars, lipids (mostly saturated fatty acids) and other metabolites. Peak labels represent assignment of NMR signals to the metabolites. Peaks numbering: 1, Lipid terminal CH_3 (0.9 ppm); 2, Lipid backbone methylene groups (1.31 ppm); 3, Lipid (–CH_2–CH_2–CO–O) (1.60 ppm); 4, Lipid (–CH_2–CH=CH–) (2.07 ppm); 5, Lipid (–CH_2–CO–O) (2.27 ppm); 6, Isobutyrate (2.38 ppm); 7, Lipid (–CH=CH–CH_2–CH=CH–) (2.77 ppm); 8, Choline (3.21 ppm); 9, Betaine (3.27 ppm); 10, d-(+)sorbose (3.6 ppm); 11, Trehalose (3.75 ppm); 12, Sucrose (3.82 ppm); 13, D-Glucosaminate (3.88 ppm); 14, Fructose (4.09 ppm); 15, d-(–) tagatose (4.19 ppm); 16, *β*-glucose (4.66 ppm); 17, Galactose (5.25 ppm); 18, *N*-acetyl-alpha-d-glucosamine (5.33 ppm); 19, *α*-Maltose + *β*-maltose (5.42 ppm).

Table 1. List of identified metabolites and their NMR chemical shift values, used for metabolite fingerprinting of wheat seeds

Metabolite	Chemical shift (ppm)
Lipid terminal CH_3	0.906
Amino acid (isoleucine, leucine, valine)	0.91
Lipid backbone methylene groups	1.318
Amino acid (alanine)	1.47
Lipid (–CH_2–CH_2–CO–O–)	1.601
Amino acid (isoleucine, lysine)	1.9
Lipid (–CH_2–CH=CH–)	2.07
Lipid (–CH_2–CH_2–CO–O–)	2.26
Amino acid (glutamine, glutamate)	2.39
Lipid (–CH=CH–CH_2–CH=CH–)	2.77
Amino acid (aspartate)	2.81
Choline	3.21
Betaine	3.27
d-(+) sorbose	3.6, 3.7
Sucrose	3.82, 5.4
D-Glucosaminic acid	3.88
D-Cellobiose	3.96
Fructose	3.56, 4.05, 4.09
Creatinine	4.02, 4.04
d-(–) tagatose	4.16, 4.19
β-Glucose	4.66
α-Glucose	3.69, 3.76, 5.22
α-Maltose	5.24
d-(+) galactose	3.83, 5.25
Lipid (–CH_2–CH=CH–)	5.33
α-Maltose + *β*-maltose (C_1 non-reducing end)	5.42

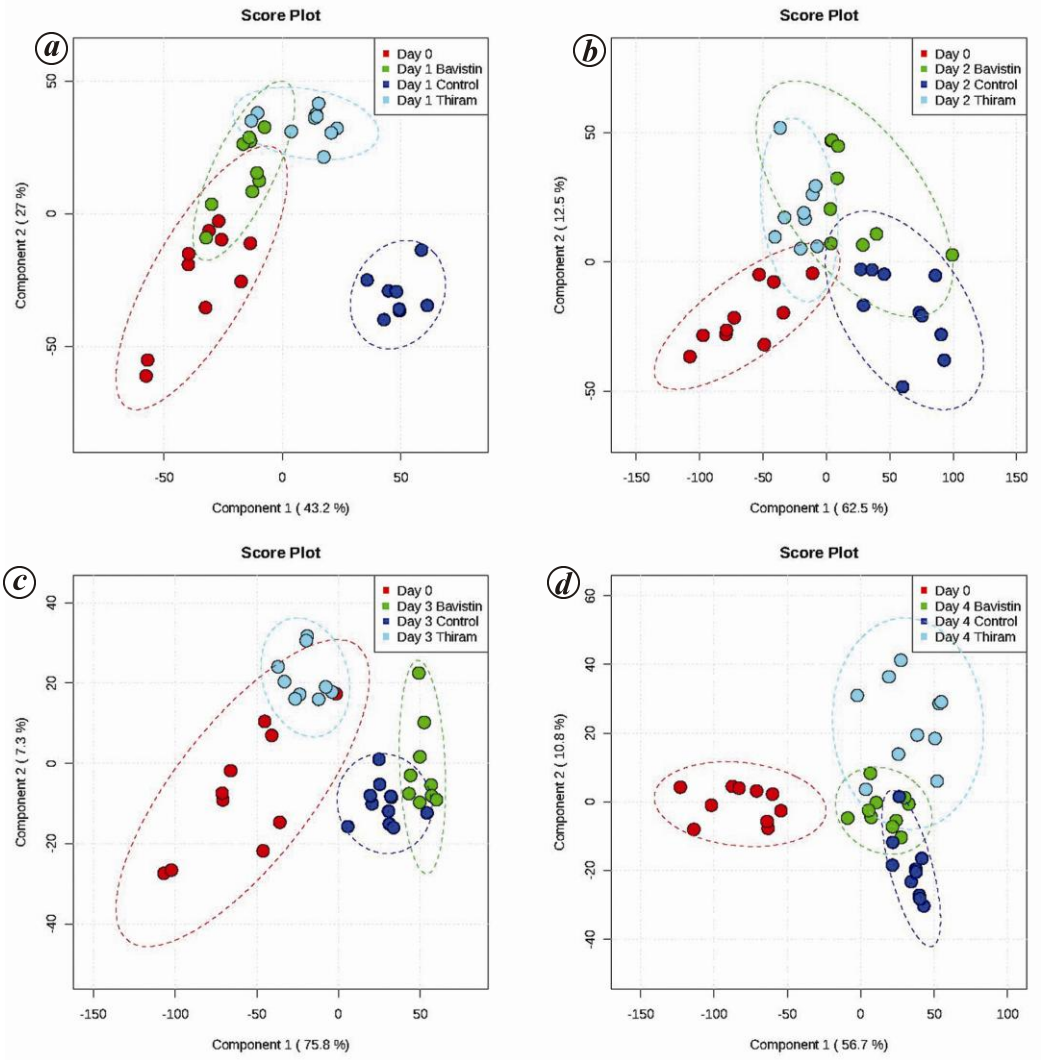

Figure 3. PLS-DA scores plot of component 1 versus component 2 for wheat seeds showing differences between control and fungicide-treated seeds during different imbibition time points as (***a***) day 1 (24 h from imbibition), (***b***) day 2 (48 h from imbibition), (***c***) day 3 (72 h from imbibition) and (***d***) day 4 (96 h from imbibition). The numbers in parentheses indicate the overall variance accounted for by the first two principal components. The ellipses represent the Hotelling T^2 with 95% confidence.

after 1000 permutation tests was $<0:001$, indicating that the PLS-DA model built is robust and credible (see Figure S8 Supplementary Material online). Similar conclusions could be drawn on repeating the PLSDA analysis by changing the reference peak to maltose instead of betaine (Figure S9, Supplementary Material online).

The loading plots help identify the major metabolites responsible for the separation between differentially treated seeds. A representative loading plot for day 3 PLS-DA score plot is shown in Figure 4. As seen from the loadings component 1 (Figure 4 *a*), on the third day of progress in the germination cycle, bavistin-treated seeds and control seeds have a higher monomeric carbohydrate content and lower lipid content than thiram-treated and day 0 (ungerminated) seeds. Betaine levels are also decreased in control and bavistin-treated seeds. Loadings component 2 (Figure 4 *b*) further separates fungicide-treated seeds from the control. Fungicide-treated seeds (both bavistin and thiram-treated) have higher contents of lipid, betaine and some carbohydrates compared to control seeds on day 3. In particular, the content of sucrose, the form in which plants uptake carbohydrates, is higher in fungicide-treated seeds, indicating sucrose is not being utilized (other sugars, for example glucose, fructose, etc. are present in lower quantities in fungicide-treated seeds). Further, the higher lipid content indicates a possibility of hindrance in metabolism. A higher betaine content indicates its osmotolerant role. All these results indicate a possible defect in lipid metabolism at the level of lipid conversion and also a defect in carbohydrate mobilization as the possible reasons behind slow germination of fungicide-treated seeds.

Wheat seeds are monocots that contain reservoirs of starch as stored carbohydrates in their endosperm[21]. The uptake of water activates the biochemistry of seeds and triggers the release of an enzyme (amylase) that increases

cellular respiration, wherein starch is transformed into sugars. As seen in Figure 5 *a*, intensity of sugar peaks increases in NMR spectra of the seeds on day 3 after imbibition, due to the fact that polymers such as starch and lipids are being converted to sugars. The NMR peak intensities (as obtained from area under the curves) of fungicide-treated seeds in Figure 5 *a* show less increase in monomers (glucose 3.69 and 3.76 ppm; fructose 3.56 and 4.05 ppm; galactose 3.76 ppm and maltose 5.24 ppm) compared to the control seeds, confirming the reduced metabolic activity of the fungicide-treated seeds. Similar conclusions can be made from Figure 6 *a–c*, where control seeds show greater increase in the carbohydrate region than fungicide-treated seeds.

Betaine (3.27 ppm) is a phytonutrient and osmolyte present in wheat seeds that improves tolerance for drought, salinity and low temperatures[22], and alleviates saline stress-induced dormancy in wheat seeds by enhancing amylase activity and improving mobilization rate of sugars[23]. The betaine peak at 3.27 ppm in Figure 5 *b* is missing in the day 3 spectra of control seeds, which confirms its metabolism on germination hypothesis. Concomitantly, the presence of this peak, but with reduced intensity in the day 3 spectra of fungicide-treated seeds, confirms the role of betaine as osmotolerant, to overcome seed dormancy and suggests the help provided by betaine in overcoming fungicidal stress.

A day-wise comparison of 1D ^{1}H spectra of wheat showing spectral differences in control and fungicide-treated seeds in the lipid region is given in Figure 5 *c*. As evidenced from the spectra, there is a decrease in the lipid content in both fungicide-treated seeds and control seeds; however, the decrease occurs at different rates. Missing resonances of 0.9, 1.31, 1.6, 2.07 and 2.27 ppm in the NMR spectra of day 3 in control wheat seeds show that the lipids have been metabolized after three days of imbibition. The reduced intensities of these peaks in day 3 NMR spectra of fungicide-treated seeds show that lipid metabolism is occurring, but has slowed down in fungicide-treated seeds. Also, comparing the two fungicides, lipid peak intensities in bavistin are lower than in thiram-treated seeds, showing that lipid metabolism in the latter is further decreased compared to the former. Conclusions are further confirmed by comparing 2D spectra of different seeds in lipid the region (Figure 6 *d–f*).

NMR experiments were performed at several imbibition time points and changes in the relative concentrations of significant metabolites (obtained as percentage of relative integrals after normalizing each integral region to the total sum of all integrals for each spectrum) with time were quantified. It can be observed from the loading plots that differences in the concentration of lipids and sugars contribute the most in differentiating between different seed types. Hence concentrations of lipids, betaine,

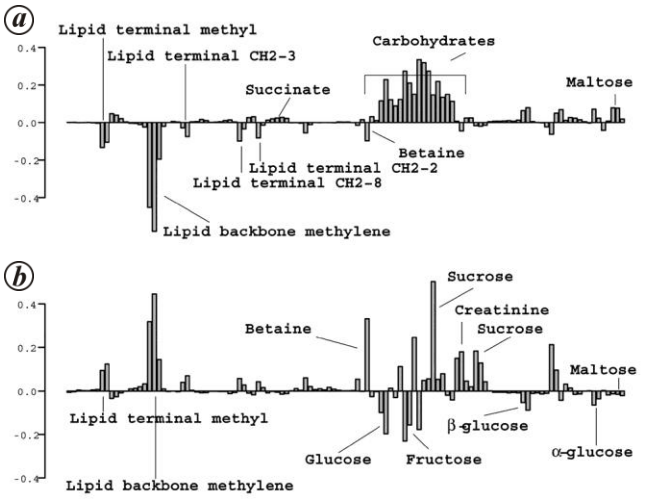

Figure 4. Loadings plot for (***a***) component 1 and (***b***) component 2 of PLS-DA model for discrimination of metabolites in control and fungicide-treated wheat seeds, based on the HRMAS-NMR spectra.

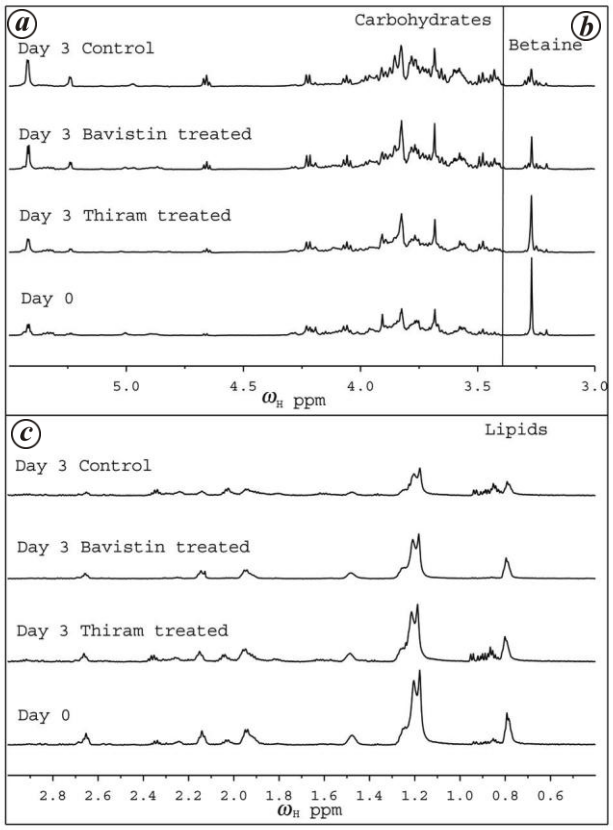

Figure 5. Comparison of (***a***) carbohydrate region (3.56, 3.88, 4.09 and 4.19 ppm), (***b***) betaine peak (3.27 ppm) and (***c***) lipid region (0.9, 1.31, 1.6, 2.07 and 2.27 ppm) from 1D ^{1}H HR-MAS NMR spectra of wheat, showing spectral changes on day 3 of imbibition in control and fungicide-treated seeds (thiram and bavistin-treated). The spectra show a decrease in peak intensity in the lipid region and of the betaine peak, and an increase in peak intensity in the carbohydrate region on day 3 in comparison to day 0. The changes are more pronounced in the control seeds compared to the fungicide-treated seeds.

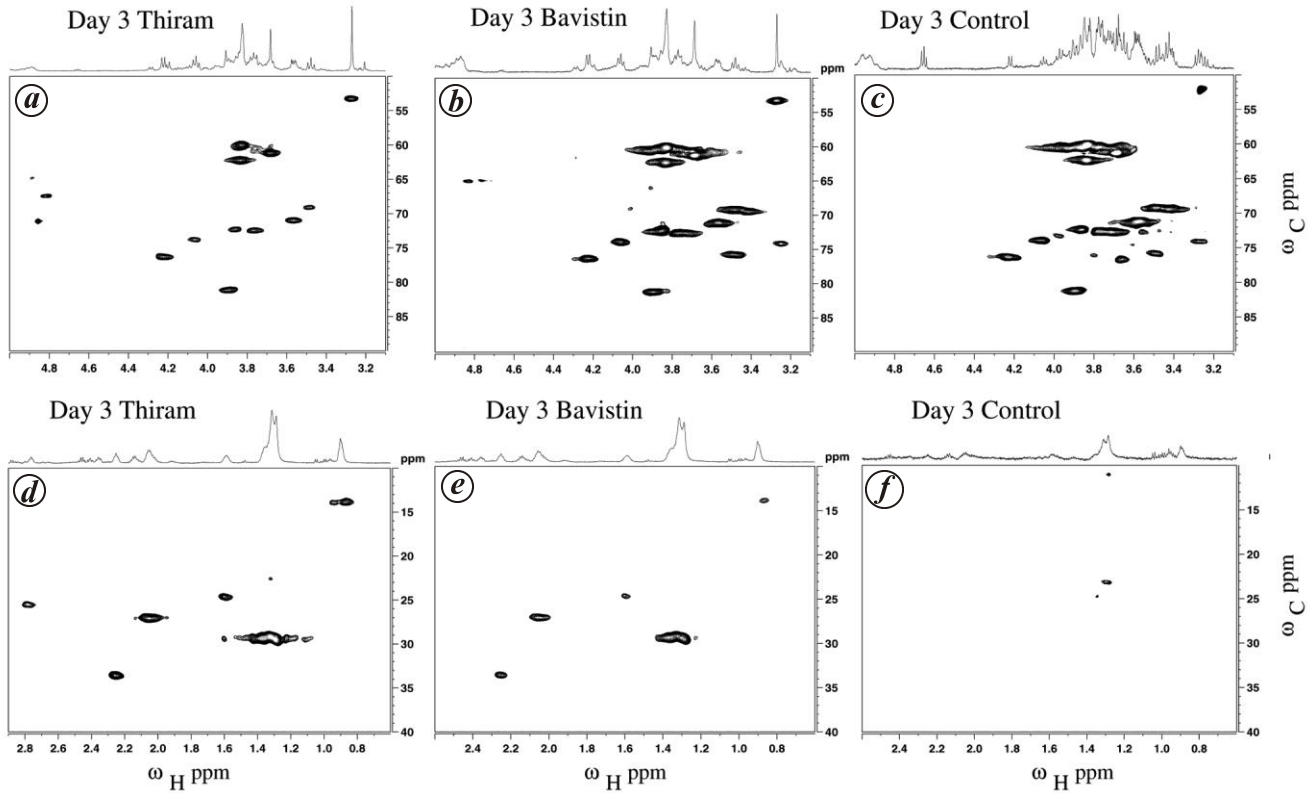

Figure 6. 2D HR-MAS ^{13}C–^{1}H HSQC NMR spectrum of wheat seeds recorded at 600 MHz. Proton chemical shifts (ω_H in ppm) and carbon chemical shifts (ω_C in ppm) are represented along the *X* and *Y* axes respectively. (Top panel) Spectra of carbohydrate region for (***a***) thiram-treated, (***b***) bavistin-treated and (***c***) control seeds. (Bottom panel) Spectra of lipid region for (***d***) thiram-treated, (***e***) bavistin-treated and (***f***) control seeds, on day 3 of imbibition.

sucrose and various monomeric sugars were monitored in different seed types (control and fungicide-treated) at regular intervals of 12 h till the 96th hour in the germination cycle (Figure 7). These time plots further support the hypothesis that metabolism is reduced in fungicide-treated seeds as the concentrations of lipids, betaine and sucrose are higher and those of monomeric sugars are lower in fungicide-treated seeds compared to control, indicating that these metabolites are being used up at a much slower rate in the fungicide-treated seeds.

Several conclusions can be drawn by comparing the day 3 NMR spectra of both control and fungicide-treated seeds shown in Figures 5 and 6 and by observing the metabolite concentrations over different imbibition times (Figure 7). Lipids and starch are converted to simple sugars and finally simple sugars are converted to sucrose to be transported to the growing embryo during seed germination. The lipids get converted to sugars and are utilized first during germination; hence lipid content decreases. The sugars are required for the growing embryo to germinate. The day 3 spectrum has more peaks in the sugar region and less peaks in the lipid region. There are two reasons for this increase in number of peaks: (i) lipids are being converted to sugars to be utilized, and (ii) polymers of sugars are breaking down into monomers and since most of the sugar peaks are present in the same region in the NMR spectrum, the number of peaks increases. Further, day 3 spectra of control seeds have fewer peaks in the lipid region and more peaks in the carbohydrate region compared to fungicide-treated seeds (since growth in control seeds is faster). Also, since growth is slower in non-systemic fungicide-treated seeds compared to systemic fungicide-treated seeds, we see fewer carbohydrate peaks in the spectra of the former compared to the latter (Figures 5 and 6; Figures S3 and S4 Supplementary Material online).

Wheat seeds consist mainly of carbohydrates in the form of starch (about 70%) that provides most of the energy for germination. Lipids have also been reported to be present in wheat seeds, though in lesser amounts than carbohydrates and are present mainly in aleurone tissue[24]. At the beginning of germination, water triggers the release of an enzyme alpha-amylase that hydrolyses starch to simple sugars. Lipids are also hydrolysed to sugars via the glyoxylate cycle. Both starch and lipids are ultimately converted to simple sugars and finally to sucrose to be transported to the growing embryo during seed germination. The various steps involved in the conversion of starch and lipids to sucrose are shown in Figure 8. The metabolites that differentiate between different seed

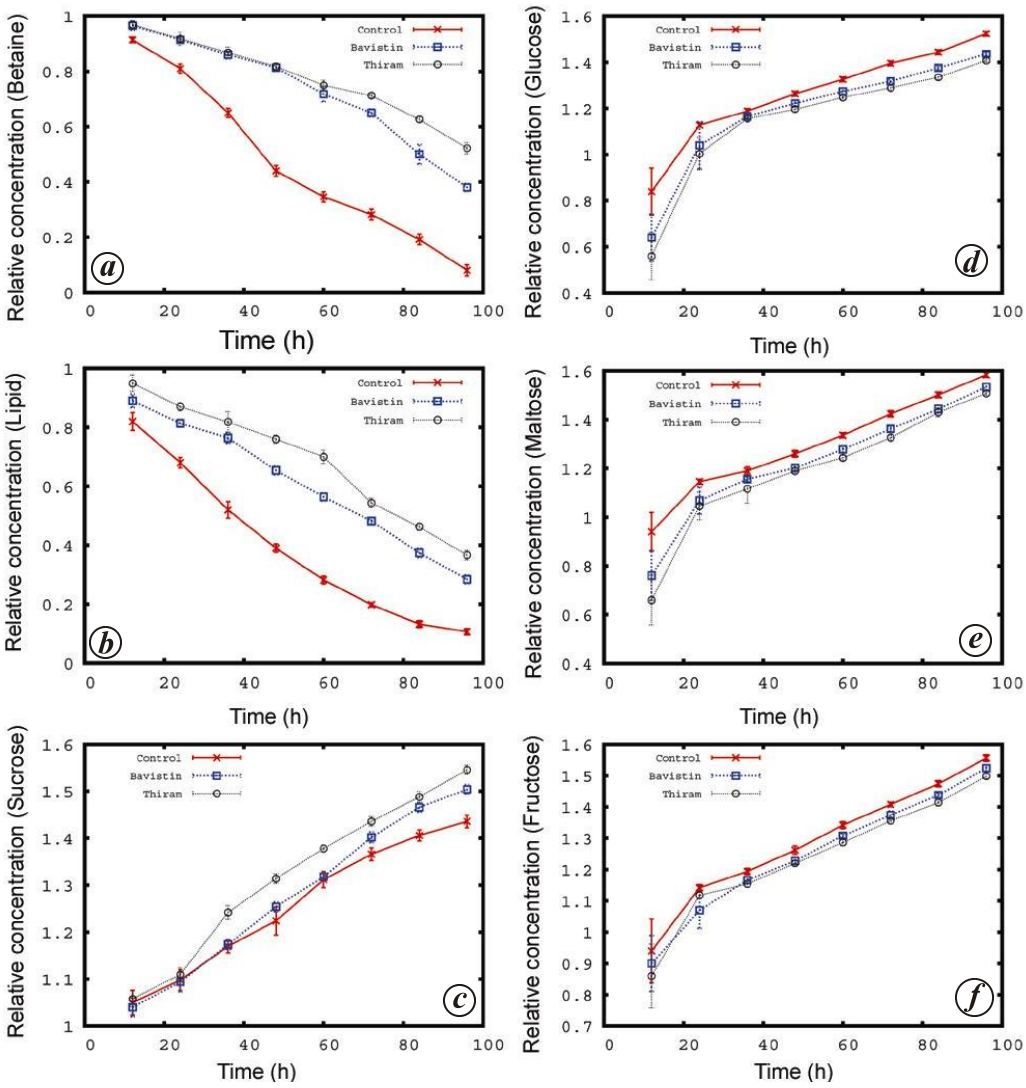

Figure 7. Plots of relative concentrations of (***a***) betaine (3.27 ppm), (***b***) lipid (1.31 ppm), (***c***) sucrose (5.4 ppm), (***d***) glucose (4.66 ppm), (***e***) maltose (5.24 ppm) and (***f***) fructose (3.56 ppm) peaks in control (cross), bavistin-treated (square) and thiram-treated (circle) wheat seeds over a time period of 96 h from the start of experiment, extracted from the HR-MAS NMR spectra. Each data point is the average of at least ten replicates, with the error bars representing the standard deviation.

treatments are fatty acids, carbohydrate monomers and sucrose. Fatty acid concentration was found to be higher in fungicide-treated seeds, indicating a slowed down conversion of lipids. Also, carbohydrate monomers were found to be present in lower concentrations in fungicide-treated seeds compared to control seeds whereas sucrose, which is synthesized from these monomers, was found to be present in higher amounts in fungicide-treated seeds. This implies two possibilities: starch not being properly converted to sugars and sucrose not being utilized efficiently by germinating wheat seeds and thus hinted at a possible defect at the level of sugar mobilization. Though less lipid conversion can be related to less carbohydrate monomer concentrations, this cannot be the sole reason for less carbohydrate monomers as lipids are present in lesser amounts compared to carbohydrates and sugar monomers are contributed mainly by starch hydrolysis. Also, the loading plots show that the metabolites such as malate (2.33 and 2.35 ppm), oxaloacetate (2.377 ppm), succinate (2.39 ppm), and acetyl-CoA (1.14 and 1.26 ppm) contribute little towards differentiating between different seed treatments. Thus, the results indicate that fungicide treatment of seeds could interfere both at the level of lipid conversion and carbohydrate mobilization, thus delaying and decreasing the growth of fungicide-treated seeds during the germination cycle.

In summary, this study showed that both systemic and non-systemic fungicides delay the germination cycle in wheat seeds, with non-systemic fungicides affecting the metabolic processes and hence the metabolite signal intensities to a greater extent than systemic fungicides. HR-MAS NMR-based metabolomics is a novel and non-invasive technique that provides a metabolomic approach to the question of phytotoxicity and growth decrease due

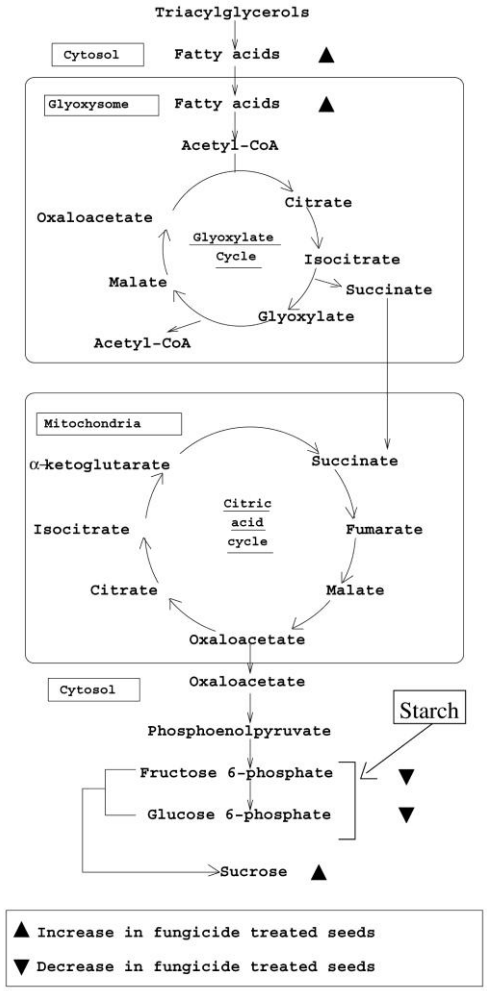

Figure 8. The metabolic pathway responsible for the conversion of lipids and starch to sucrose in germinating seeds. Triangles show the relative increase (or decrease) in metabolite concentration in fungicide-treated seeds compared to control seeds.

to fungicide treatment of seeds. It is expected that plant metabolite quantification using HR-MAS NMR methods will contribute significantly toward progress in the fields of plant physiology and chemical ecology.

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