An *In Vivo* ^{31}P NMR Study of the Phosphorus Metabolites in Developing Seeds of Wheat, Soybean and Mustard

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***In vivo* ^{31}P NMR spectra of wheat, soybean and mustard seeds were recorded during ripening. Signals were detected from phosphomonoesters, cytoplasmic and vacuolar inorganic phosphate (P_i), phytate, nucleoside triphosphate (NTP) and nucleoside diphosphosugars. The spectra of extracts showed an accumulation of phytate during development, accompanied by a decrease in inorganic phosphate, and this was reflected in the *in vivo* spectra. The intrinsic width of the resonances was a significant obstacle to the interpretation of the *in vivo* spectra in all three cases, and the problem became more severe with increasing maturity. However, it was still possible to use the chemical shift of the cytoplasmic P_i signal to monitor cytoplasmic pH in both soybean and mustard, and the results provide evidence for the existence of a hypoxic state in developing seeds during the active biosynthetic phase.**

Magn. Reson. Chem. **35**, S125–S132 (1997) No. of Figures: 10 No. of Tables: 0 No. of References: 30

Keywords: NMR; *in vivo* ^{31}P NMR; anaerobiosis; cytoplasmic pH; hypoxia; developing seeds

Received 2 May 1997; revised 15 August 1997; accepted 15 August 1997

INTRODUCTION

Plant tissues have been studied extensively by non-invasive NMR methods[1] and a significant proportion of this work has been conducted on seeds.[2] Thus, low-field ^{1}H NMR has proved to be invaluable in the determination of the oil and moisture contents of many agronomically important seeds,[3] natural abundance ^{13}C NMR provides a convenient way to determine the fatty acid composition of oilseeds[4–7] and high-resolution NMR imaging is capable of generating both spatially resolved analytical information and morphological detail.[8] The emphasis of much of this work is on generating analytical data non-destructively for plant breeding purposes,[3,7] but it is also possible to use NMR to monitor responses to physiological and developmental events. Seed germination and seed maturation are the obvious targets for such studies, since both processes involve extensive metabolic and morphological rearrangement. In fact, germination has been extensively investigated by both NMR imaging[8–10] and *in vivo* NMR spectroscopy,[11–13] whereas maturation or ripening has attracted considerably less interest.[14] The low moisture content and heterogeneity of many seeds can lead to some relatively unfavourable NMR properties, and although this can limit the interpretation of both imaging and spectroscopic data, it is still possible to use these methods to generate useful information.

Insufficient access to atmospheric oxygen, arising from the poor permeability of the outer layers of the seed coat to gaseous exchange,[15,16] could lead to hypoxia in developing seeds during active biosynthesis.[17] This could have implications for crop productivity, reducing the final grain weight,[17] but direct evidence for a hypoxic state in developing seeds is lacking. Hypoxia usually leads to acidification of the cytoplasm and this phenomenon can often be investigated using *in vivo* ^{31}P NMR.[18] A similar approach could be useful for investigating the putative hypoxic state in developing seeds, and this paper reports a series of experiments in which *in vivo* ^{31}P NMR spectra were recorded from developing seeds of wheat, soybean and mustard. These three species were chosen as being representative of three types of crops: starch accumulating (wheat), protein accumulating (soybean) and oil accumulating (mustard).

EXPERIMENTAL

Plant material

Seeds of mustard (*Brassica juncea* cv. Varuna), soybean (*Glycine max.* cv. PK 564) and wheat (*Triticum aestivum* cv. Sel.111) were soaked in aerated water overnight and then germinated in the dark in 0.1 mM $CaSO_4$ at 25 °C. After 48 h the germinated seeds were sown in pots containing peat and compost. The plants were maintained

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Contract grant sponsor: Agricultural and Food Research Council; *Contract grant number*: LRG 11.

 CCC 0749–1581/97/SI0125–08 $17.50

in a greenhouse at 25 °C with an extended day length of 16 h at the Department of Plant Sciences, Oxford, and they were watered as required. The flowers were tagged on the day of anthesis as a measure of the maturity of plants. The developing seeds were harvested at approximately weekly intervals and immediately taken to the laboratory for recording the NMR spectra. The fresh and dry weights (80 °C) of the seeds were also recorded. Equivalent seed samples were frozen in liquid nitrogen and stored at −20 °C prior to measurement of the ^{31}P spectra of their extracts.

Perchloric acid extraction

Freeze-dried seed samples (0.3–0.4 g) were ground in a cooled pestle and mortar with 3 ml of 1 M perchloric acid at 4 °C and the slurry obtained was centrifuged at 3000 *g* for 10 min to give a clear solution. A 100 μmol amount of cyclohexane-1,2-diaminetetraacetic acid was added to the solution and the pH was adjusted to 6.5 with 3 M K_2CO_3. The solution was centrifuged at 3000 *g* for 10 min to remove precipitates, defatted with 5 ml of $CHCl_3$, passed through an Amicon ultrafiltration membrane (YM5) under a pressure of 50 lb in^{-2} (340 kPa) to remove solutes with a molecular weight greater than 5000 Da and then passed down a Chelex column (Sigma) to remove paramagnetic ions. The filtrate was freeze-dried and stored at −20 °C. The lyophilized powder was dissolved in 50 mM 2-[*N*-morpholino]ethanesulphonic acid + 5 mM EDTA (pH 5.5) for measurement of NMR spectra.

Estimation of volatile reducing substances including ethanol

Wheat and mustard plants were grown in pots filled with sandy loam soil and maintained under normal agronomic practices in an open field at the Indian Agricultural Research Institute, New Delhi. Developing grain samples were crushed slightly inside a chilled pear-shaped flask having an inlet for N_2 and an outlet end dipped in a tube containing 3 ml of alkaline $KMnO_4$ solution (20 μg of $KMnO_4$ + 100 ml of 0.2% NaOH). The seeds were killed by immersing the flask in a boiling water-bath and N_2 was bubbled through slowly (1 ml min^{-1}) for the first 8 min and then rapidly (10 ml min^{-1}) for a further 2 min. Control experiments were carried out by omitting the seeds from the flask. The permanganate solution was diluted with 3 ml of distilled water and the absorbance was recorded at 600 nm. The total content of volatile reducing substances was expressed as μg of ethanol per mg fresh weight using a calibration graph for ethanol.

NMR measurements

^{31}P NMR spectra were recorded at 121.49 MHz on a Bruker CXP spectrometer using a double tuned $^{13}C/^{31}P$ 10 mm diameter probehead. The freshly harvested seed samples were restricted to the volume within the receiver coil and an oxygenated 0.5 mM $CaSO_4$ solution was circulated though the NMR tube at a rate of about 5 ml min^{-1} using the circulation system described elsewhere.[19] A capillary containing 70 mM phosphocreatine was used to provide a reference signal at −2.44 ppm relative to 85% H_3PO_4, and spectra were recorded immediately after the transfer of the seeds to the NMR tube to minimize any effect from the onset of rehydration. ^{1}H-decoupled ^{31}P NMR spectra were accumulated using a 45° pulse angle, a recycle time of 0.5 s and a total acquisition time of at least 30 min. These acquisition conditions were considered to be optimum on the basis of the expected T_1 and T_2* values.[20] The signal-to-noise ratio was enhanced using a 16 Hz exponential multiplication and the number of data points in the FID was increased from 1K to 16K by zero filling prior to Fourier transformation. Resolution-enhanced spectra were obtained using Gaussian multiplication. Cytoplasmic pH values were calculated from the chemical shift of the cytoplasmic P_i signal using a calibration graph described elsewhere.[21]

Proton decoupled ^{31}P NMR spectra of seed extracts were obtained in files of 1024 scans, each containing 2K data points, using a 90° pulse angle, a 4 s recycle time, a line broadening of 4 Hz and zero filling to 16K. The resonances were assigned by comparison with previously published spectra and by spiking with phytate or P_i solution at pH 5.5.

RESULTS AND DISCUSSION

The moisture content of the developing seeds decreased with increasing maturity (Fig. 1) and wheat seeds had a relatively lower moisture content than mustard and soybean seeds throughout their development. Numerous NMR studies on germinating seeds have shown that the resolution of the spectra improves as hydration proceeds, and so the increase in linewidth of the soybean ^{31}P NMR signals during maturation shown in Fig. 2 is entirely consistent with previous observations. At a high moisture content [Fig. 2(a)] the soybean spectrum resembles the ^{31}P NMR spectra of other plant

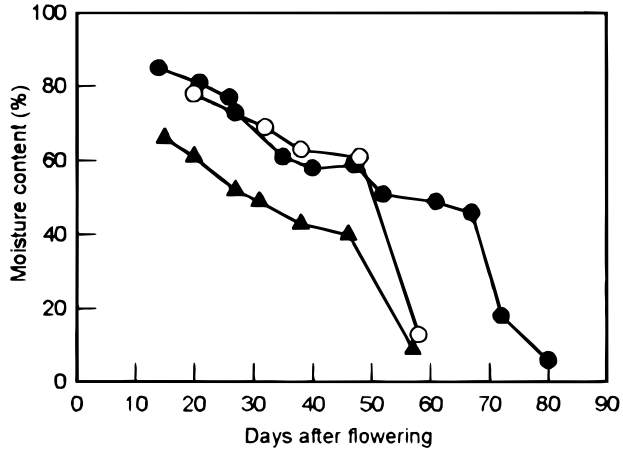

Figure 1. Moisture content of developing mustard (●), soybean (○) and wheat (▲) seeds. Watering was stopped at 46 (▲), 48 (○) and 67 (●) days after flowering.

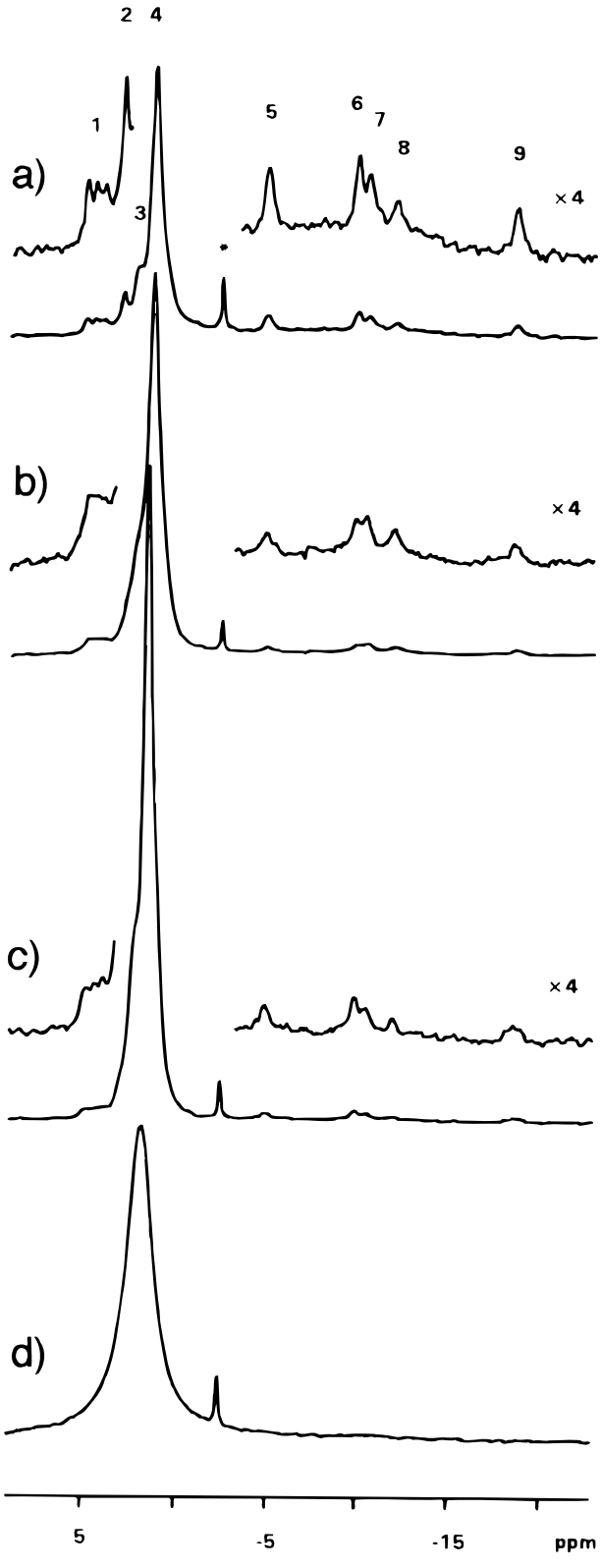

Figure 2. ^{31}P NMR spectra of intact soybean seeds, (a) 20, (b), 32, (c) 48 and (d) 58 days after flowering. The numbered peaks may be assigned as follows: 1, several phosphomonoesters; 2, cytoplasmic P_i; 3, vacuolar phytate; 4, vacuolar phytate and vacuolar P_i; 5, 6 and 9, the γ-, α- and β-phosphates, respectively, of nucleoside triphosphate; 7, UDP-glucose and NAD(P)(H); and 8, UDP-glucose. The signal from the phosphocreatine chemical shift reference is marked with an asterisk.

tissues, e.g. maize root tips,[22] and most of the signals can be assigned simply by comparison with the published spectra. However, the extra resonance at 2.2 ppm [peak 3, Fig. 2(a)] together with the increased width of the signal at 1.2 ppm [peak 4, Fig. 2(a)] indicates that the latter cannot be assigned entirely to vacuolar P_i and that the spectrum includes a contribution from phytate. This storage compound has been detected in seeds[12,23] and potato tubers[23] and its presence in the soybean spectra was confirmed by preparing perchloric acid extracts (Fig. 3). The extract spectra showed the characteristic 1:2:2:1 intensity distribution expected for *myo*-inositol hexakisphosphate phosphate at mildly acidic pH values[24] and the assignment of the spectra was confirmed by spiking with phytate and P_i (data not shown). In fact, the position, number, relative intensity

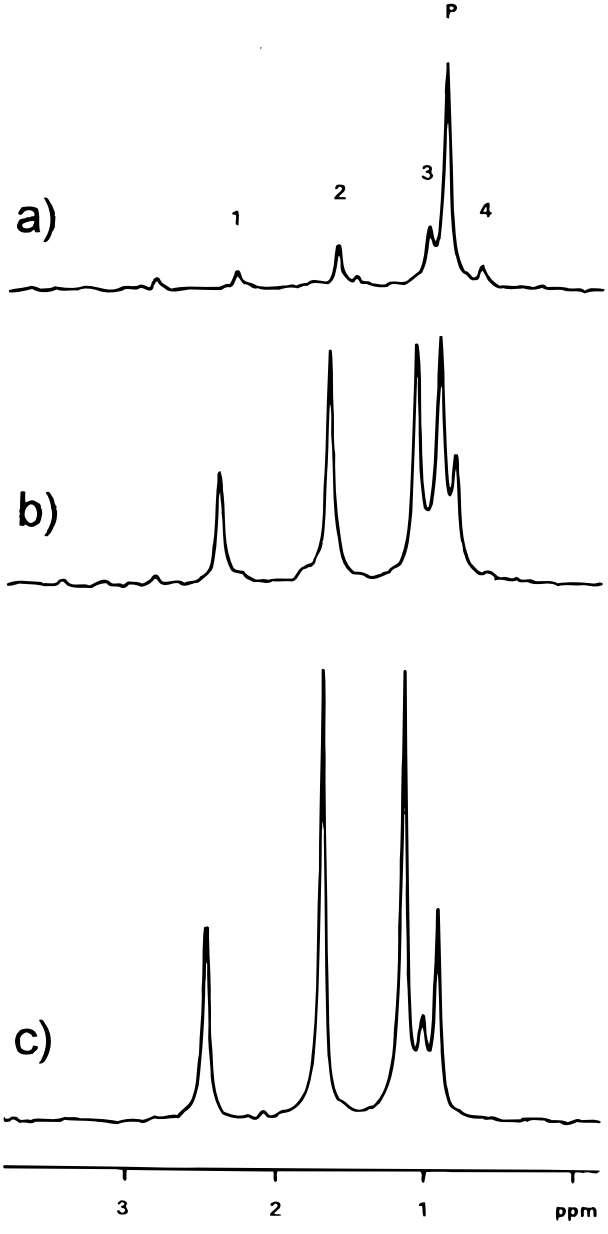

Figure 3. ^{31}P NMR spectra of perchloric acid extracts of soybean seeds, (a) 20, (b) 32 and (c) 48 days after flowering. The numbered peaks are assigned to phytate and the signal labelled P comes from P_i.

and linewidth of the phytate signals are all strongly dependent on pH, ionic strength and binding to metal ions, with the result that there is rarely an exact correspondence between the intensity distribution observed *in vitro* and *in vivo*, but the data in Figs 2 and 3 are consistent with the detection of vacuolar phytate in the *in vivo* spectra (Fig. 2).

Figure 2 shows that increasing maturity led to (i) a gradual loss of resolution across the spectrum, (ii) a large increase in intensity in the vacuolar P_i/phytate region, (iii) a downfield shift in the position of the dominant signal [peak 4, Fig. 2(a)] and (iv) a reduction in the contribution from the cytoplasmic signals [peaks 1, 2 and 5–9, Fig. 2(a)]. The changes in the intensity and position of the signals in the 1.2–2.2 ppm region of the spectrum are consistent with the synthesis and accumulation of phytate during ripening. This interpretation is confirmed by the extract spectra (Fig. 3), which show that the dominant component of the extract changed from P_i at 20 days after flowering to phytate at 48 days after flowering. Most of the phytate intensity occurs downfield of the P_i signal and this accounts for the downfield shift in the dominant signal observed *in vivo* (Fig. 2). As regards the other spectroscopic changes in Fig. 2, the deterioration in the resolution during maturation can be attributed to the reduced moisture content (Fig. 1) and to the build-up of insoluble storage material that can interact with the remaining pools of soluble metabolites, while the loss of the cytoplasmic signals is consistent with the increased vacuolation and decreased metabolic activity in the fully ripened seed.

The resolution and definition of the seed spectra in Fig. 2 are considerably better than those reported in earlier NMR studies of the germination[11] and maturation[14] of soybeans. This can be attributed to the higher magnetic field strength used here (7.05 rather than 2.35 T) and to the utilization of a circulating oxygenated medium to maintain the seeds in a good physiological state during the measurements. The resulting improvement in the spectral quality makes resolution enhancement a practical proposition, and Fig. 4 shows a series of spectra obtained using the Gaussian multiplication algorithm.[25] Resolution enhancement of the P_i/phytate region of the spectrum allows the definition of the cytoplasmic P_i signal (peak 2, Fig. 4) in the spectra from seeds with a moisture content as low as 60%, and this suggests that it should be possible to use the spectra to measure the cytoplasmic pH of the seeds at various stages of development.

Mustard seeds showed a similar time course to soybean seeds during maturation (Fig. 5) and again the spectral quality was sufficiently good to measure to chemical shift of the cytoplasmic P_i signal in resolution-enhanced spectra (Fig. 6). The *in vivo* time course showed that the best resolution was obtained at a moisture content of around 70%, which corresponds to the active phase of triglyceride synthesis,[26] and that the resolution subsequently deteriorated as dehydration proceeded. Phytic acid accumulated in much the same way as in soybean, with a concomitant downfield shift in the position of the dominant signal in the ^{31}P NMR spectra.

In contrast to soybean and mustard, wheat seeds gave poorly defined spectra even at fairly high moisture

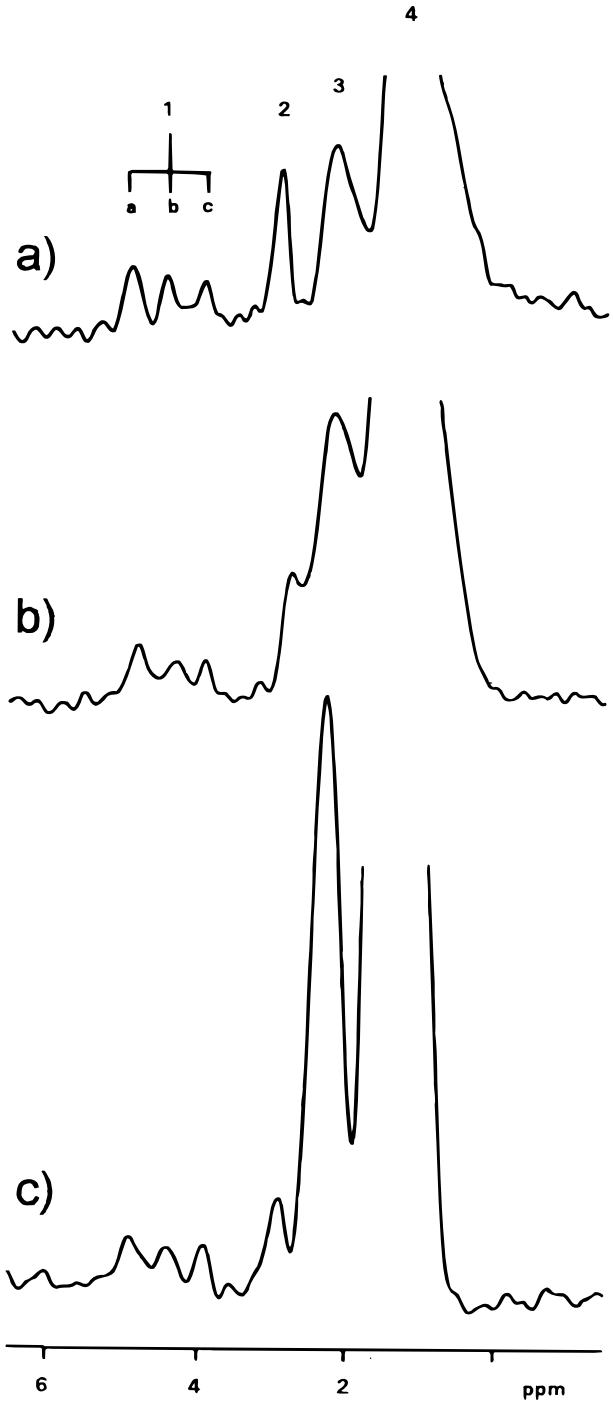

Figure 4. Resolution-enhanced ^{31}P NMR spectra of intact soybean seeds, (a) 20, (b) 32 and (c) 48 days after flowering. The labelled peaks may be assigned as follows: 1, several phosphomonoesters, including glucose-6-phosphate (1a) and phosphocholine (1c); 2, cytoplasmic P_i; 3, vacuolar phytate; and 4, vacuolar phytate and vacuolar P_i. The chemical shift of the cytoplasmic P_i signal is 2.90 ppm in (a), 2.72 ppm in (b) and 2.91 ppm in (c).

contents (Fig. 7), and it was not possible to resolve the cytoplasmic P_i signal in resolution-enhanced spectra (data not shown). Although rapid water loss, resulting in a water content of only 66% at 15 days after flowering (Fig. 1), may be a contributory factor to the poor resolution, a comparison of Fig. 7(a) (wheat; 66% moisture content) with Fig. 2(b) (soybean; 69% moisture

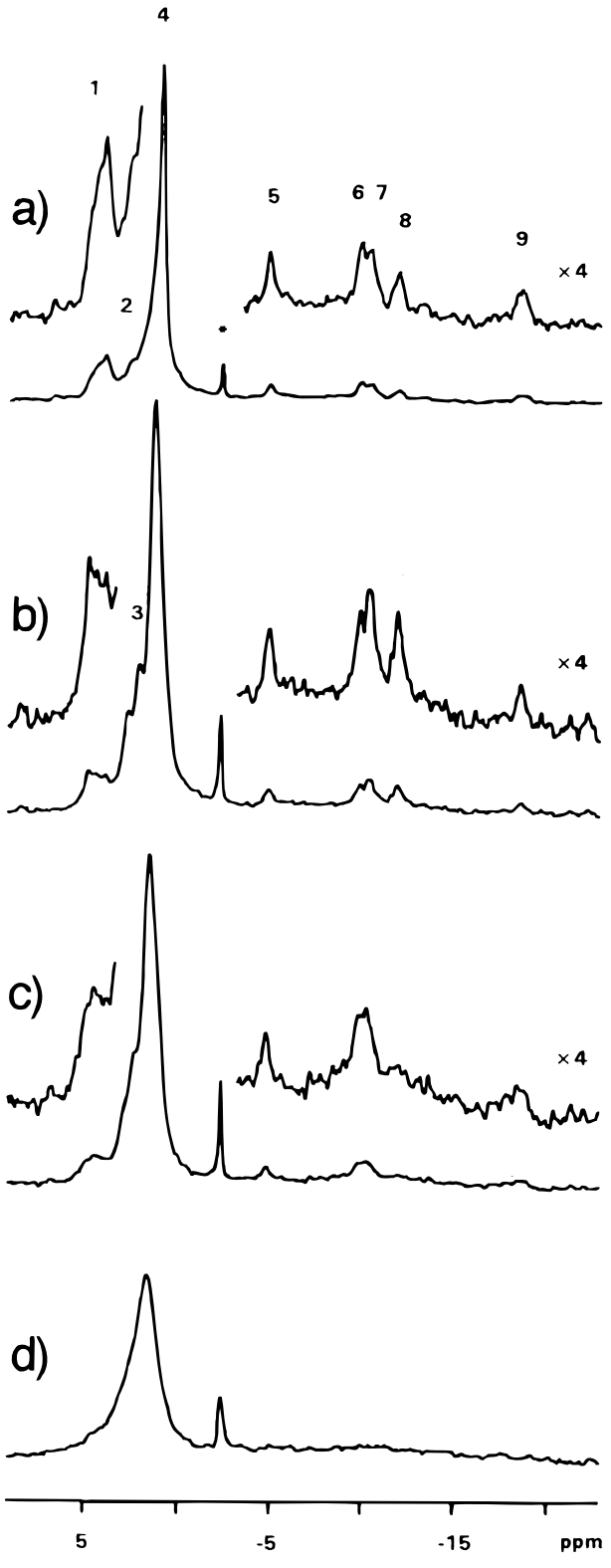

Figure 5. ^{31}P NMR spectra of intact mustard seeds, (a) 14, (b) 27, (c) 40 and (d) 72 days after flowering. The peak assignments are the same as in Fig. 2.

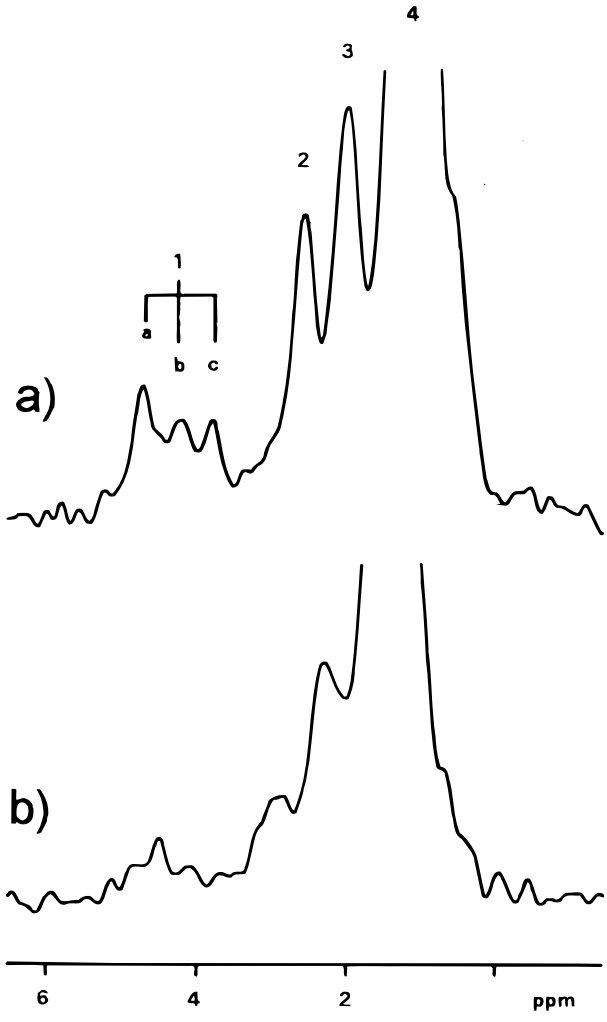

Figure 6. Resolution-enhanced ^{31}P NMR spectra of intact mustard seeds, (a) 27 and (b) 61 days after flowering. The peak assignments are the same as in Fig. 4. The chemical shift of the cytoplasmic P_i signal is 2.56 ppm in (a) and 2.90 ppm in (b).

content) and Fig. 5(c) (mustard; 58% moisture content) indicates that the resolution of the wheat spectrum was relatively poor despite the comparable water contents. Furthermore, the very broad signal observed at the end of the ripening process [Fig. 7(d)] indicates that the phosphorus metabolites became immobilized in the dry seed, and it may well be that immobilization occurs to a greater extent in a starch-accumulating seed such as wheat than in the protein-accumulating soybean and oil-accumulating mustard seeds. Hence seed water content and the nature of the storage products may both be important in determining the quality of the ^{31}P NMR spectra from developing seeds.

Phytate again increased during maturation, but the retention of a vacuolar P_i signal at 0.8–0.9 ppm (peak 4, Fig. 7) in the spectrum from seeds with a moisture content of only 43% [Fig. 7(c)] contrasted with the situation in soybean and mustard where the stored P_i pool declined rapidly as phytate increased. This interpretation of the *in vivo* spectra was confirmed by the extract spectra (Fig. 8), which showed a significant quantity of P_i in the presence of strong phytate signals [Fig. 8(c); contrast Fig. 4(c)]. In oilseeds phytate is largely concentrated in protein bodies in the cells of the radicle and the cotyledon,[27] whereas in cereals it accumulates in the protein bodies of the aleurone layers.[28] It is possible that differences in the ripening process and the site of phytate accumulation are responsible for the observed differences in the seed

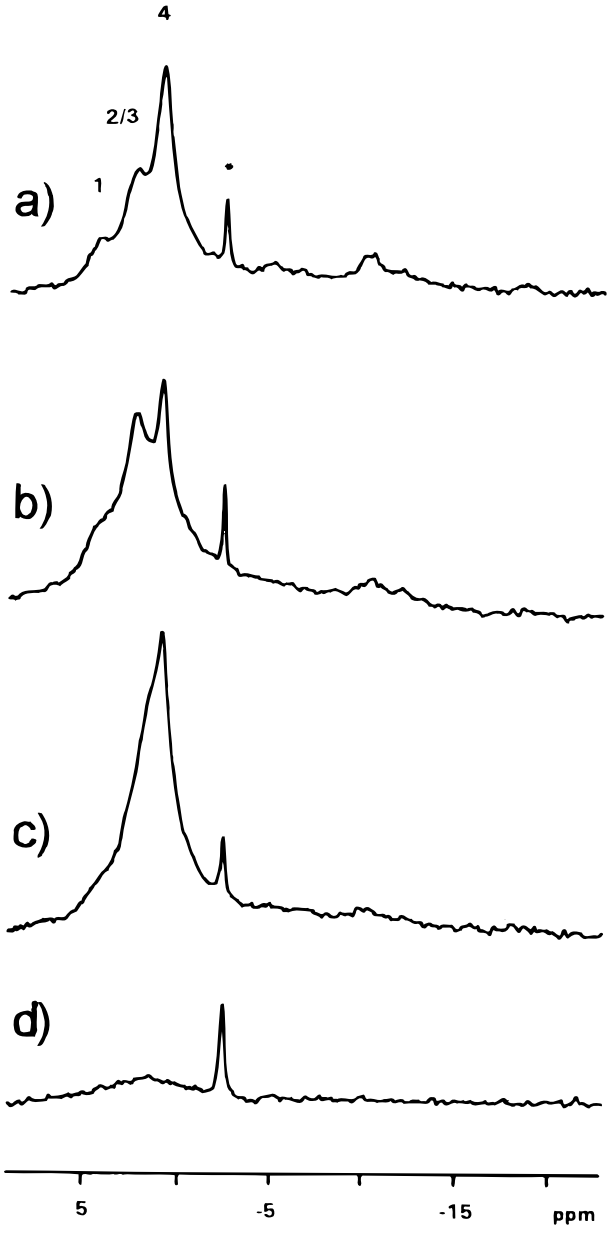

Figure 7. ^{31}P NMR spectra of intact wheat seeds, (a) 20, (b) 27, (c) 38 and (d) 57 days after flowering. The peak assignments are the same as in Fig. 2.

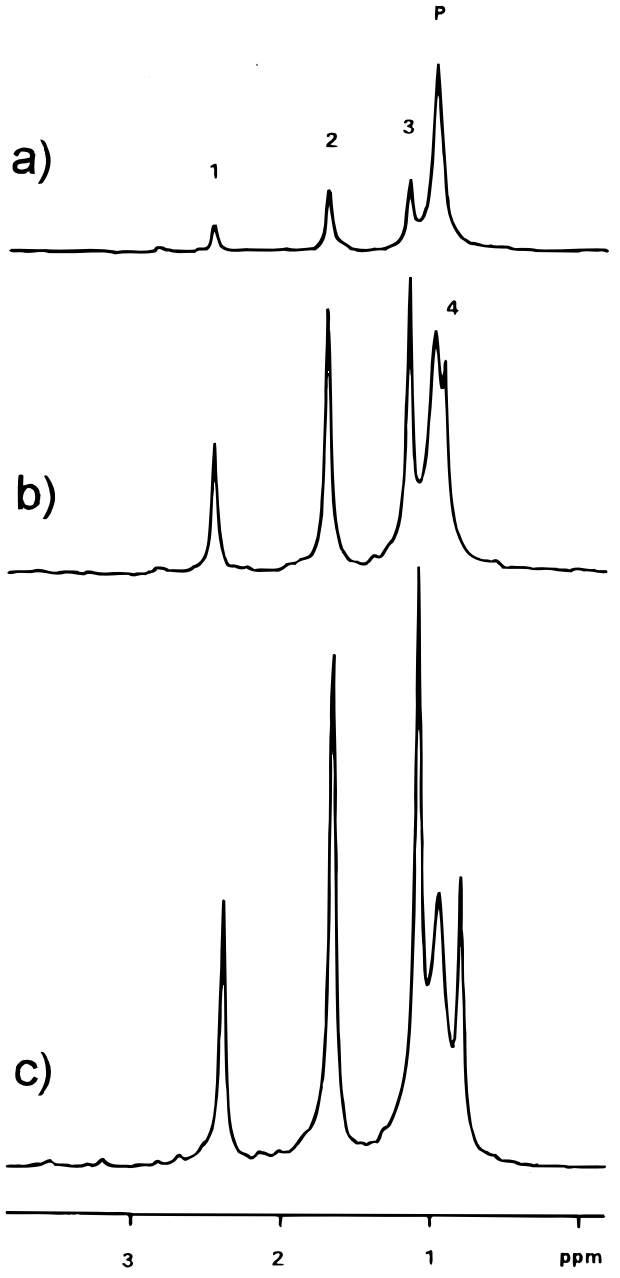

Figure 8. ^{31}P NMR spectra of perchloric acid extracts of wheat seeds, (a) 15, (b) 20 and (c) 38 days after flowering. The peak assignments are the same as in Fig. 3.

spectra, i.e. for the persistence of the vacuolar P_i signal and the broader lines in the wheat spectra, but it is difficult to draw a firm conclusion on the significance of these observations.

The resolution-enhanced spectra of soybean (Fig. 4) and mustard (Fig. 6) showed that the cytoplasmic P_i signal could be resolved over a wide range of moisture contents, enabling the cytoplasmic pH to be monitored during maturation (Fig. 9). In the case of soybean, with only limited data, the cytoplasmic pH fell by about 0.2 pH units at around 30 days after flowering, before recovering to 7.6, and in the case of mustard the cytoplasmic pH gradually increased from an initial value of 7.2 to reach a value of 7.6 at around 50 days after flowering. Acidification of the cytoplasm is commonly observed in plant tissues during oxygen deprivation[18] and Fig. 9 provides direct evidence for the existence of a

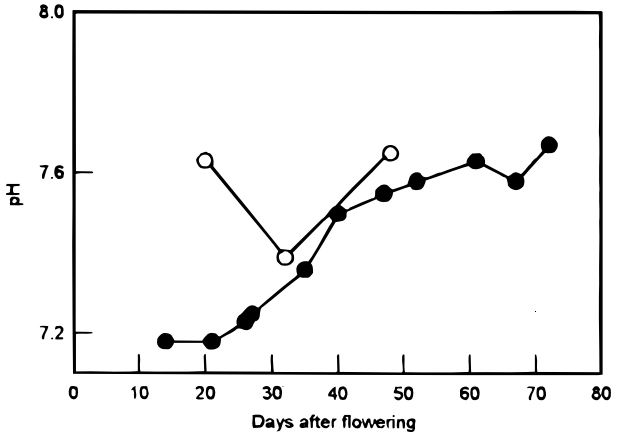

Figure 9. Cytoplasmic pH of developing mustard (●) and soybean (○) seeds.

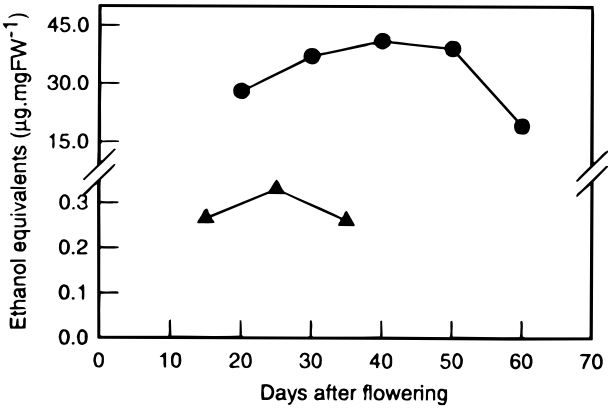

Figure 10. Content of volatile reducing compounds, expressed in ethanol equivalents, in developing mustard (●) and wheat (▲) seeds.

hypoxic state during the maturation of both soybean and mustard seeds. Biosynthesis is at its most active at around 30 days after flowering, and it seems likely that the hypoxic state reflects the rapid consumption of oxygen to support ATP synthesis coupled with the slow diffusion of oxygen through the outer layers of the seeds.[15] It may also be significant that oilseeds require more photosynthate, and therefore more energy, than protein-storing legumes to yield the same seed weight,[29] and this may be a contributory factor in the more prolonged hypoxic state in mustard (Fig. 9).

Some support for the existence of a hypoxic state *in vivo* was provided by *in vitro* data on the reducing substances that accumulated in the seeds during maturation (Fig. 10). The assay measured the volatile reducing compounds in the seed, including the ethanol generated in hypoxic tissues, and the data are expressed in ethanol equivalents. Hypoxia should lead to an increase in ethanol production, and Fig. 10 shows that the accumulation of volatile reducing compounds went through a maximum during the maturation of both wheat and mustard seeds. While this is consistent with the presence of hypoxic tissues in the developing seeds, it should be noted that the very simple assay procedure used here is non-specific and that low molecular weight fatty acids and the degradation products of glucosinolates are likely to be significant contributors to the much larger pool of volatile reducing compounds in the mustard seeds. To avoid this problem, and to strengthen the evidence for the hypoxic state, future work should use an enzymic assay, such as the method described by Bernt and Gutmann,[30] to explore the correlation between the pH changes observed by NMR (Fig. 9) and ethanol production in the developing seeds.

CONCLUSION

It has been shown that interpretable *in vivo* ^{31}P NMR spectra can be recorded from developing seeds, and that these spectra can be used to monitor changes in metabolism and energy status during the initial stages of maturation. Although there was inevitably a severe loss of resolution during ripening, it was still possible to resolve the cytoplasmic P_i signal over a range of moisture contents in two of the three species examined. This allowed the determination of the metabolically important cytoplasmic pH value, providing direct evidence for the existence of a hypoxic state in developing soybean and mustard seeds. A more detailed investigation of this observation would be justified, including consideration of the extent to which factors other than pH might influence the chemical shift of the cytoplasmic P_i signal, since an understanding of the metabolic events that occur during seed development is important in assessing limitations to crop productivity and since a shortage of oxygen in a developing seed could have important implications for grain yield. The observations reported here are also of interest in showing how an NMR technique that has been largely used for studying plant nutrition and the metabolic responses of plant tissues to environmental stress can also address a question that is relevant to food science.

Acknowledgements

R.G.R. acknowledges the financial support of the Agricultural and Food Research Council (Grant LRG 11) and P.N.G. thanks the Department of Biotechnology, Ministry of Science and Technology, New Delhi, for the award of an Overseas Associateship to work at the University of Oxford. The authors also thank Dr C. M. Spickett for help in the preparation of the seed extracts and Mr A. R. Fernie for assistance with the preparation of the figures.

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