Title:

An enzymatic fluorescent assay for the quantification of phosphite in a microtitre plate format

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Short title: Enzymatic fluorescent assay for phosphite

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Abstract

A sensitive fluorometric assay for the quantification of phosphite has been developed. The assay uses the enzymatic oxidation of phosphite to phosphate by a recombinant phosphite dehydrogenase with NAD^+ as co-substrate to produce the highly fluorescent reaction product resorufin. The optimised assay can be carried out in a 96-well microtitre plate format for high-throughput screening purposes and has a detection limit of 0.25 nmol phosphite. We have used the method to quantify phosphite levels in plant tissue extracts and to determine phosphite dehydrogenase activity in transgenic plants. The assay is suitable for other biological or environmental samples. As phosphite is a widely used fungicide to protect plants from pathogenic oomycetes, the assay provides a cost-effective and easy-to-use method to monitor the fate of phosphite following application.

Keywords:

Phosphite, phosphite dehydrogenase, PtxD, resorufin, oomycete

Introduction

Phosphite (Phi, $H_2PO_3^-$), the anionic form of phosphorous acid (H_3PO_3), is a reduced form of phosphate and is used to protect plants from pathogens in agriculture and natural ecosystems [1; 2]. Recently a protective effect against parasitic nematodes has also been observed in wheat and oat [3]. Phosphite is especially effective in controlling the spread and infection process of important oomycete phytopathogens such as *Phytophthora* and *Pythium* spp. *Phytophthora infestans* is the causative agent of the potato blight disease that led to the Irish potato famine in 1845. *P. sojae* and *P. capsici* are important pathogens of crop plants and together *Phytophthora* spp. cause agronomic losses of several billion US dollars per year [4]. Other species such as *P. cinnamomi* and *P. ramorum* have devastating effects in natural ecosystems, causing 'dieback disease' and 'sudden oak death', respectively [5; 6].

Phosphite is marketed widely as a fungicide (e.g. as the active compound in products such as fosetyl-Al/Aliette or Agrifos), but also rather controversially as a fertiliser. This latter use has recently been questioned as the fertilising effect is likely caused by oxidation of phosphite to phosphate by soil microbes, while phosphite itself is rather detrimental to growth in plants with low phosphorus status [1; 7]. The ability of phosphite to control disease in plants is based on a complex mode of action. At higher concentrations it directly inhibits the growth of the pathogen by interfering with metabolic processes, whereas at lower concentrations it has an indirect effect by activating plant defence responses by inducing gene expression and enhancing pathogen recognition [1; 2]. Although phosphite is not metabolised in plants, it interferes with their phosphate-sensing mechanisms, presumably by mimicking phosphate and competing with phosphate for uptake by phosphate transporters. Phosphite treatment of phosphate-deficient plants leads to the suppression of the phosphate-starvation response, i.e. the activation of genes or morphological adaptations that are used by plants to obtain phosphate from nutrient-poor soils [8; 9]. Phosphite has a similar effect in yeast and oomycetes [10; 11].

Dissecting the effects of phosphite in plants and pathogens requires the ability to quantify phosphite with high sensitivity in biological and non-biological samples. Several methods, such as high pressure ion chromatography (HPIC) and gas chromatography-mass spectrometry (GC-MS), have been developed to measure phosphite in environmental samples (for review see [12; 13]) and similar approaches have been used to quantify phosphite in plant tissues [14; 15; 16]. In recent years, nuclear magnetic resonance (NMR) spectroscopy has also been applied to analyse the distribution of phosphite and phosphate at the subcellular level [17; 18]. These methods have the disadvantage of relying on expensive instrumentation and a high-degree of user knowledge to

operate them and analyse the data. The costs per sample are also relatively high, inhibiting the measurement of larger sample sets. Here, we present an enzymatic assay for quantifying phosphite that is both sensitive and amenable to a high-throughput microtitre plate format. The assay is based on the oxidation of phosphite to phosphate by the enzyme phosphite dehydrogenase (PtxD) from *Pseudomonas stutzeri* WM88 with NAD^+ as co-substrate. The PtxD protein has been well characterised for its kinetic properties and a mutant recombinant form of PtxD with improved expression characteristics has been isolated which makes it especially well suited for this assay [19; 20]. The released reaction product NADH is quantified in a cycling assay that ultimately leads to the formation of fluorescent resorufin. Costs for this assay are low and the assay of dozens of samples can be carried out in parallel within 2 hours. We show the versatility of the assay by quantifying phosphite within plant tissues and analysing enzyme activity in transgenic plants.

Materials and methods

Materials

All chemicals were at least analytical grade (Sigma-Aldrich Australia Pty. Ltd, Sydney, Australia). A potassium phosphite standard solution was prepared by neutralising 0.1 M phosphorous acid to pH 7 with 5 M KOH. Recombinant His-tagged phosphite dehydrogenase (6xHis-PtxD) was purified as described [21] with some modifications. After harvesting *Escherichia coli* from 400 ml LB medium after induction overnight at 25 °C the pellet was resuspended in 10 ml 50 mM MOPS[1], pH 7.3; 200 mM NaCl; 10 mM β-mercaptoethanol; 30 mM imidazole; 15% glycerol; 0.1 mM PMSF and cells lysed by ultrasonication on ice. The lysate was cleared by centrifugation at 14000 g and 4 °C for 15 min before application to an affinity column (HiTrap Chelating HP column, GE Healthcare Australia Pty. Ltd., Rydalmere, Australia). The column was washed with 5 ml of 50 mM MOPS, pH 7.3; 200 mM NaCl; 10 mM β-mercaptoethanol; 50 mM imidazole; 15% glycerol and the recombinant 6xHis-PtxD eluted with 5 ml of 50 mM MOPS, pH7.3; 200 mM NaCl; 10 mM β-mercaptoethanol; 250 mM imidazole; 15% glycerol. After buffer exchange to 50 mM MOPS, pH 7.3; 200 mM NaCl; 10 mM β-mercaptoethanol; 15% glycerol using a desalting column (D-Salt Excellulose, ThermoFisher Scientific Australia Pty Ltd, Scoresby, Australia) the enzyme was stored in aliquots at -80 °C.

Plant material

Arabidopsis thaliana wild-type plants and transgenic lines (both in Col-0 background) were grown on ½ MS medium containing 0.8 % (w/v) micro agar (Duchefa, Haarlem, The Netherlands) and potassium phosphite as stated under short-day conditions (8 h light/ 16h dark) at 22 °C in a growth chamber [22].

Plant tissue extractions

Phosphite was extracted from approximately 50 mg plant tissue with 1 % acetic acid (1:10 w/v) using a tissue lyser (Qiagen Australia Pty. Ltd., Doncaster, Australia) for 1 min at 25 Hz. After clearing the lysate by centrifugation at 14000 *g* and 4 °C for 15 min, the supernatant was assayed for phosphite. For protein extraction, approximately 100 mg plant tissue was disrupted in 100 µl 50 mM MOPS, pH 7.3; 250 mM NaCl, 1 mM EDTA; 0.1 mM PMSF and 1x protease inhibitor cocktail (Sigma-Aldrich Australia Pty. Ltd, Sydney, Australia) using a tissue lyser followed by centrifugation at 14000 *g* and 4 °C for 15 min. The supernatant was applied to a desalting column

(Zeba Spin column, ThermoFisher Scientific Australia Pty Ltd, Scoresby, Australia) and protein eluted with 50 mM MOPS, pH 7.3; 250 mM NaCl, 1 mM EDTA). Protein concentrations were determined by the method of Bradford [23].

Phosphite analyses

A standard curve was produced using 0 to 20 nmol phosphite in a total of 50 µl water in individual wells of a 96-well microtitre plate. Unless stated otherwise, the reaction was started by adding 200 µl assay mix to give a final concentration of 50 mM MOPS, pH 7.3; 100 µM NAD^+; 100 µM phenazine methosulfate; 100 µM resazurin; and 1 µg recombinant 6xHis-PtxD per well. The microtitre plates were incubated at 37 °C for 1 h in the dark or product formation was directly monitored in real time. The reaction product resorufin was quantified with a fluorescence reader (DTX880 Multimode detector, Beckman Coulter Australia Pty. Ltd., Gladesville, Australia) at 535 nm excitation and 595 nm emission wavelengths. Fluorescence is given in arbitrary units (A.U.) derived after a 0.1 s signal integration period. For the determination of phosphite in plant extracts, three reactions per sample were set up in 96-well microtitre plates. These tests were sample only (25 µl plant extract + 25 µl MQ water), internal standard control (25 µl plant extract + 25 µl 0.6 mM potassium phosphite (i.e. 15 nmol phosphite), and a blank (25 µl plant extract + 25 µl MQ water). For calculation of the recovery rate for the added standard, fluorescence of the internal standard control was subtracted from that of the sample only to determine the standard amount accounted for experimentally. In the blank, PtxD enzyme was omitted to allow correction for autofluorescence of the plant extract and non-specific resazurin reduction. For determination of enzymatic activities in transgenic plants 50 µg total plant protein was used and final assay conditions were: 50 mM MOPS, pH 7.3; 1 mM NAD^+; 100 µM phenazine methosulfate; 1 mM potassium phosphite; 100 µM resazurin. High pressure ion chromatography was performed as described [14].

Results and Discussion

Development of a fluorescent assay for phosphite quantification

Several assays for the determination of NADH content in plant tissues have been developed [24]. The reduction of resazurin to the fluorescent resorufin has been used as the basis for a highly sensitive fluorescent method to measure NADH in free solution [25]. We therefore reasoned that the oxidation of phosphite to phosphate by NAD^+-dependent phosphite dehydrogenase would provide the means to measure phosphite in a microtitre plate-based assay where NADH re-oxidation is coupled to resazurin reduction using phenazine methosulfate as electron transfer agent (Fig. 1). Using such a cycling assay has the advantage that a relatively low NAD^+ concentration can be used, preventing accumulation of NADH, which is a strong inhibitor of PtxD [19].

In fluorescent assays, an inner filter effect can result from absorbance of the excitation wavelength by the dye. This leads to a decrease in assay sensitivity at elevated substrate concentrations. We therefore determined the optimal amount of resazurin for the sensitive quantification of phosphite in the assay by varying the resazurin concentration while keeping phosphite at a constant low concentration to ensure complete substrate conversion. The fluorescence initially increased with increasing resazurin in the reaction but peaked at about 50 µM resazurin and decreased with further increases in resazurin (Fig. 2). From these results we decided to use the slightly higher than optimal concentration of 100 µM resazurin in later assays to avoid early substrate depletion and thus be able to measure as high as 25 nmol phosphite in the assay. Under our optimised reaction conditions we were able to observe the reaction in real time and with high sensitivity (Fig. 3A). With the maximum of 25 nmol phosphite in the assay the formation of the reaction product resorufin reached saturation within 45 min of the start of the reaction and the fluorescent signal was constant thereafter. For the quantification of phosphite we prepared a standard curve by varying the amount of phosphite in the assay. The assay is linear up to at least 25 nmol phosphite, corresponding to a concentration of 100 µM phosphite per 250 µl assay (Fig. 3B). The lower detection limit of the assay is approximately 0.25 nmol per assay corresponding to a measureable lower sample concentration of 5µM. Published HPIC methods for the detection of phosphite in plant samples have similar detection limits and and mass spectrometry gives slightly higher sensitivity (2.5-fold) but with a need of sample pre-purification (see [12] for review). These methods are also more time-consuming taking 20 to 30 minutes per sample run on the instruments. In comparison, our microtiter plate-based assay allows the measurement of about 30 samples within one hour.

Validation of the enzyme assay

With the assay optimized assay conditions we validated the results obtained for plant tissue samples by comparison to an established method for phosphite quantification using high pressure ion chromatography (HPIC) [14]. In the course of this validation, it was apparent that for some plant samples the phosphite concentration determined by the enzymatic assay was much lower than that measured by HPIC. We reasoned that this might be caused by the presence of inhibitors in the extracts of the recombinant PtxD. To test this, phosphite was added as an internal standard to the samples. The determined phosphite concentration for these spiked extracts was less than expected based on the amount of phosphite added to the extracts (Table 1). Recoveries mainly ranged from 50 to 90 %, but we also found recoveries in the range of only 20 % in samples that had been stored for extended periods at -20 °C (Table 1, samples #9 and #10). Therefore, to account for the possibility of PtxD inhibition, a known amount of phosphite must be added to each sample to act as an internal standard for the reliable determination of the concentration in plant extracts. After correcting for PtxD inhibition, the enzyme-based and the HPIC-based assays give very similar results for extracts from plants treated with varying phosphite concentrations (Fig. 4). The differences in analyte determination between the two methods were within a range of 15%. Given that the microtitre plate-based enzymatic assay takes only about 2 h to complete, the processing of 32 samples in parallel by this method is much faster and uses less complicated instrumentation at a much lower cost than the HPIC method. We have not investigated the possible cause of PtxD inhibition by plant extracts. Repeated freeze/thaw cycles and extended storage seem to aggravate the inhibition, which is not detected in fresh extracts. It is therefore likely that chemistry within the extracts produces an inhibitory factor. Known inhibitors of PtxD activity are sulfite and arsenite [19]. It is possible that sulfite is released through degradation of sulfur containing metabolites such as cysteine, glutathione or glucosinolates, which are a major class of secondary metabolite in plants.

Application of the enzyme assay

Further tests ensured the applicability of the enzymatic assay for the measurement of phosphite in plant extracts in physiological experiments. Phosphite is taken up and distributed throughout the plant by phosphate transporters [17; 18; 26]. Intracellular phosphite is then compartmentalised further between the cytosol and organelles [18]. *Arabidopsis thaliana* was grown on medium containing varying amounts of potassium phosphite allowing the uptake of phosphite by the roots and its transport to the shoot. We then determined the phosphite concentration of the shoot tissue with the fluorescent assay. As expected, we found an increasing concentration of phosphite in the

analysed tissues with a higher availability of phosphite in the medium (Fig. 5). A concentration as low as 0.1 mM potassium phosphite in the medium was sufficient to produce tissue concentrations measurable with the assay. Further experiments with transgenic *A. thaliana* lines expressing a transgene encoding PtxD (O. Berkowitz & P.A. O'Brien, unpublished) were performed. The fluorescent assay is ideal to measure PtxD activities in those plants. After extracting total protein from the transgenic plants, the ability of the expressed PtxD protein to oxidise phosphite was measured as the production of fluorescent resorufin (Fig. 6). From the initial slopes of these enzyme kinetics the specific activity of PtxD was calculated to be 1.5 nmol $\cdot mg^{-1} \cdot min^{-1}$ for line 1 and 0.5 nmol $\cdot mg^{-1} \cdot min^{-1}$ for line 2. A higher specific activity of PtxD in line 1 is in agreement with a higher expression of the protein in this line as determined by immunoblotting (data not shown). Extracts from non-transgenic control plants lacked detectable phosphite dehydrogenase activity. This result further supports the contention that plants are unable to metabolise phosphite, leading to its accumulation within plant tissues [26; 27].

Conclusions

We have developed a sensitive assay for measuring phosphite in plant tissues. Given the use of phosphite as fungicide and purported fertiliser in agricultural and natural ecosystems, the availability of a cost-effective and easy-to-use assay will enable monitoring of phosphite application and its accumulation in treated plants and the environment. This will also allow a better understanding of how phosphite protects plants from pathogenic oomycetes and induces defence responses at the molecular physiological level. The assay also has potential for determining phosphite levels in soil and other material and for quantifying the enzymatic capacity of microbial populations to oxidise phosphite.

Acknowledgments

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[1] Abbreviations used:

EDTA, ethylenediaminetetraacetic acid; HPIC, high pressure ion chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; MS, Murashige and Skoog; PMSF, phenylmethylsulfonyl fluoride; PtxD, phosphite dehydrogenase; FW, fresh weight

Tables

Table 1: Recovery of an internal phosphite standard

Each plant extract was spiked with 15 nmol phosphite (Phi) as an internal standard (Std.) and measured against unspiked extracts to calculate the recovery rates as the measured difference between both assays against the actual known amount of standard added. The derived recovery was then used to calculate the adjusted amount of Phi in the extracts. Samples marked with an asterisk (*) had been stored for an extended period at -20 °C and been subjected to repeated freeze/thaw cycles, leading to partial inhibition of the enzymatic activity.

Sample	Phi extract [nmol][a]	Recov. Phi Std. [nmol][a]	Recovery [%]	Adj. Phi extract [nmol]
#1	1.7	11.7	78.0	2.2
#2	0.9	7.9	52.7	1.8
#3	1.9	11.3	75.1	2.5
#4	7.9	11.8	78.9	10.0
#5	6.3	12.6	83.7	7.5
#6	10.4	11.6	77.2	13.4
#7	8.1	13.4	89.0	9.1
#8	9.7	13.4	89.5	10.9
#9*	2.9	2.9	19.2	15.0
#10*	3.0	3.7	24.6	12.2

[a] Values derived from a standard curve of assay fluorescence versus phosphite.

Figures

Figure 1

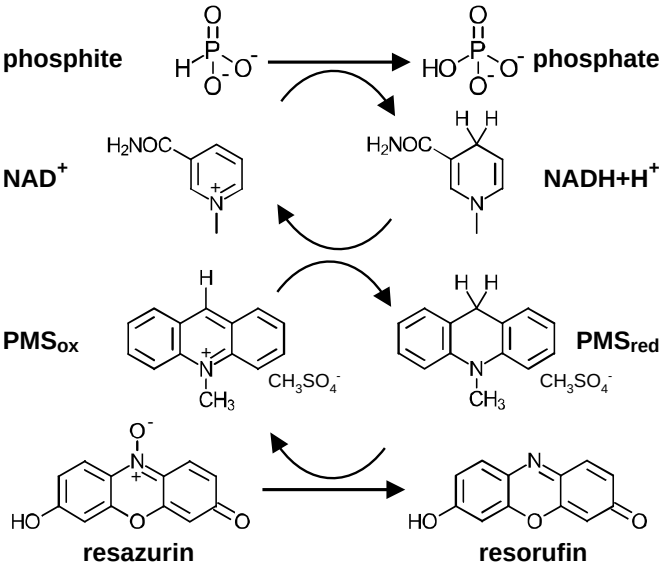

Figure 2

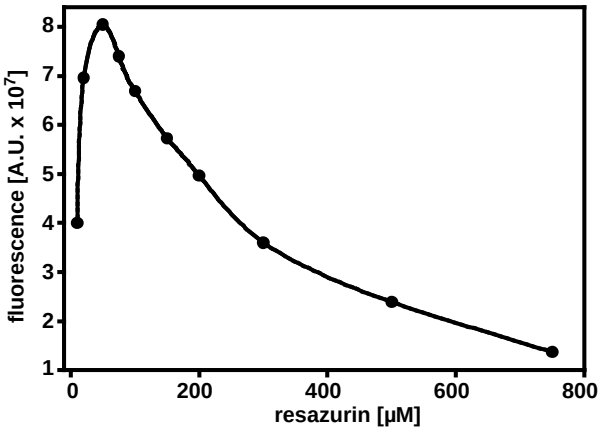
fluorescence [A.U. x 10^7]
8
7
6
5
4
3
2
1
0
200
400
600
800
resazurin [µM]

Figure 3

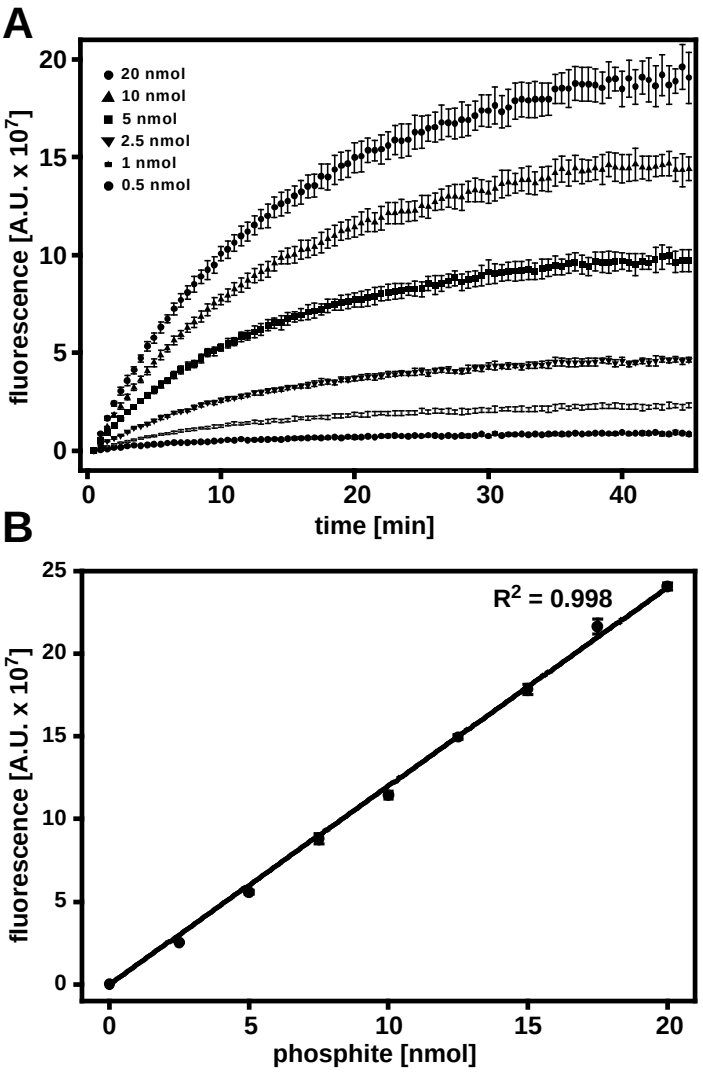
A
20 nmol
10 nmol
5 nmol
2.5 nmol
1 nmol
0.5 nmol
fluorescence [A.U. x 10^7]
time [min]
B
R^2 = 0.998
fluorescence [A.U. x 10^7]
phosphite [nmol]

Figure 4

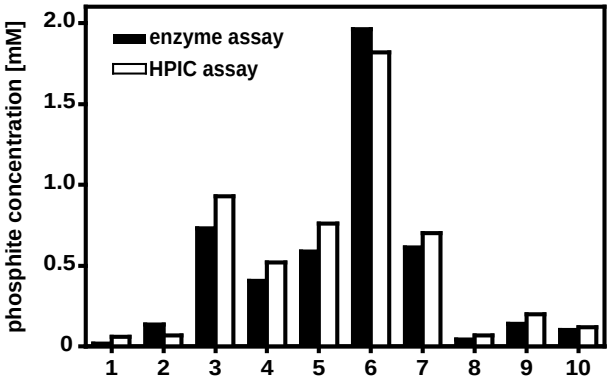
phosphite concentration [mM]
2.0
1.5
1.0
0.5
0
enzyme assay
HPIC assay
1
2
3
4
5
6
7
8
9
10

Figure 5

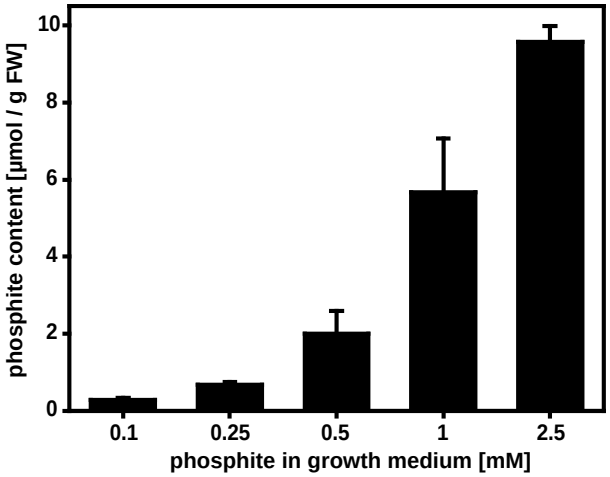
phosphite content [µmol / g FW]
10
8
6
4
2
0
0.1
0.25
0.5
1
2.5
phosphite in growth medium [mM]

Figure 6

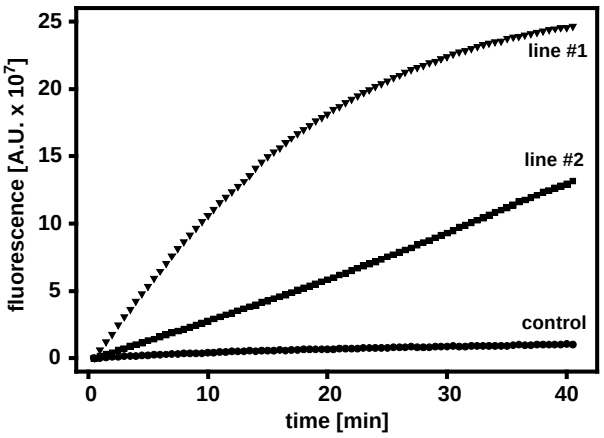
25
20
15
10
5
0
fluorescence [A.U. x 10^7]
0
10
20
30
40
time [min]
line #1
line #2
control

Figure legends

Figure 1: Schematic representation of the assay reaction

Phosphite dehydrogenase (PtxD) converts phosphite (Phi) to phosphate (Pi) with NAD^+ as the co-substrate. In a cycling reaction, electrons are transferred from NADH via phenazine methosulfate (PMS_{red}) to reduce resazurin to the final reaction product resorufin which is highly fluorescent at a wavelength of 590 nm with an excitation wavelength of 535nm.

Figure 2: Optimisation of resazurin concentration in the assay

Standard assay conditions were used with 5 nmol phosphite per assay and with increasing resazurin concentrations. Fluorescence was measured after a one-hour incubation to allow for full oxidation of phosphite and resazurin. The signal peaked at an assay concentration of about 50 µM resazurin and then declined due to the internal filter effect.

Figure 3 Optimisation of assay conditions

(A) Real-time detection of resorufin production in the enzyme assay. Increasing amounts of phosphite were used. Shown are means ± SE (n=3). (B) Standard curve for the quantification of phosphite. For each end-point determination, the reaction was incubated at 37 °C for 1 h to ensure completion of the reaction. A conversion factor of $8.33 \cdot 10^{-8}$ nmol phosphite / A.U. was calculated from the linear regression. Shown are means ± SE (n=3).

Figure 4: Comparison of results from the enzymatic assay and high pressure ion chromatography

The phosphite concentration of randomly chosen extracts of phosphite-treated *Arabidopsis thaliana* plants (samples #1 to #10) were determined by both the enzymatic assay and high pressure ion chromatography. Values for the enzyme assay were corrected for enzyme inhibition by calculating the recovery of a spike of phosphite used as an internal standard.

Figure 5: Phosphite accumulation in *Arabidopsis thaliana* plants

Plants were grown on solid medium containing 0.5 mM phosphate and varying amounts of phosphite. Plant shoots were homogenised and extracted before using the fluorescent assay to measure tissue phosphite concentrations. Given are means ± SE (n=3).

Figure 6: PtxD activity in transgenic *Arabidopsis thaliana* plants

Transgenic plants expressing a microbial PtxD protein were grown on solid medium for 3 weeks and 100 mg of pooled tissue from each line was used for further analyses. The PtxD activity in three lines was measured using 50 µg extracted and desalted total protein in the fluorescent assay. Line 1 showed a higher specific enzyme activity than line #2, whereas the non-transgenic wild type control had no detectable activity. Specific activities were calculated from the initial slopes (0 to 5 mins) of the curves.