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Original Research

Nitrogenous Nutrition Affects Uptake of Arsenic and Defense Enzyme Responses in Wheat

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Abstract

Nitrogenous nutrition influences the availability of other plant resources and, consequently, affects plant defense responses. Both a shortage and excess of N impact plants´ ability to accumulate and survive metals/metalloids, but available data are still fragmented and often contradictory. A series of 8 different NH_4NO_3 concentrations, ranging from zero to excessive nitrogen (35 mM N), was applied in growth media to hydroponically grown wheat (*Triticum aestivum*). The plants were grown at a sublethal concentration of arsenic (5 mM As^{3+}) for 10 days and foliar accumulation of As, N and P was determined. In addition, induction of defense-related chitinase and β-1,3-glucanase enzyme isoforms was quantified upon the separation of plant protein extracts in polyacrylamide gels. As^{3+} interfered with N and P accumulation in shoots and strongly activated several enzyme isoforms. These responses varied with the N supply and indicated a low rate of As accumulation at low N concentrations. On the other hand, limited As transfer to shoots was a clear benefit at high N concentrations. Nevertheless, both extreme N concentrations restricted the growth. Several enzyme isoforms of both chitinases and β-1,3-glucanases exerted sensitivity to As^{3+}, N supply or both. Their individual responses, however, contradict the generally accepted view on positive correlation between these defense molecules and N nutrition. Impacts of interplay between As^{3+} toxicity and nutritional

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stress on wheat responses are discussed. The results might contribute to knowledge applicable in efficient fertilization and food safety strategies.

Keywords: inducible defense, glucanhydrolases, metalloid toxicity, PR-proteins, trade-off

Introduction

Both nitrogen (N) shortage and excess severely affect plants. When acting for longer periods, N starvation mainly restrains the synthesis of nucleic acids and enzymes [1]; on the other hand, stagnant nutrient solutions exceeding 10 mM nitrate decelerates plant growth [2, 3]. Compared to N shortage, N toxicity is rare in nature, however, over-fertilization of agricultural soils has become a serious environmental problem in recent decades. For some plant species, a threshold of 25 mM N has been determined for toxicity [4 and therein].

A growing body of evidence suggests that the nutritional status of plants not only limits plant growth but also determines the availability of resources for defense. Data indicate that nitrate transporters affect the outcomes of different plant-pathogen interactions by mediating ROS production, regulating salicylic acid (SA) signaling towards the resistance by safeguarding energy, and/or by accumulating certain metabolites [5,6]. N addition to plants seems to generally reduce the content of secondary metabolites such as e.g. the diverse groups of phytoalexins [7], while the accumulation of other compounds such as terpenes or alkaloids showed more variable correlations with N supply [8]. Since many of these compounds possess antimicrobial activity, it is not surprising that excessive N often results in compromised resistance against microbial pathogens [9]. However, opposite responses have been reported for antimicrobial proteins, albeit the impact of N availability on inducible defense components has been relatively poorly studied, especially in relation to abiotic stress. Several studies on plant responses to different fertilizer regimes confirmed (often unwittingly) the N impact on individual defense-related enzymes such as chitinases [10-12], peroxidases [8] and β-1,3-glucanases [13]. Generally, the activities of these enzymes correlated positively with excessive N supply [8, 10], but recently much more peculiar, N-specific responses have been demonstrated in wheat grown within a broad range of N supply/availability [3]. In the last study, the behaviour of the individual isoforms probably reflected a variable NO_3^- sensitivity and/or efficiency of different nitrate transport systems in plants [14]. More importantly, it suggests possible consequences to the defense ability of plants [3].

Arsenic (As) is a toxic metalloid element that enters natural geochemical processes, but due to different anthropogenic activities (e.g. mining, use of pesticides and fertilizers etc.), it increasingly contaminates soils and water and poses serious health risk to plants as well as animals and humans [15, 16]. Though it can be beneficial at very low soil contents [17], at higher concentrations it is generally toxic to plants and hampers many physiological processes. Arsenic enters the plants in sap-mobile As^{3+} or As^{5+} forms through transporters of other elements (mainly P and Si) and through water-channel proteins [18]. While As^{5+} is readily reduced to As^{3+} as a first step of detoxification, in non-hyperaccumulating plants, the reduced As^{3+} is the main As form that may be sequestered to vacuoles following complexation with phytochelatins and subsequent transport *via* ABC (ATP Binding Cassette) transporters [19]. Recently, inositol transporters (INTs) have been recognized to perform As loading to phloem [20].

Early after exposure, As triggers an unavoidable burst of deleterious oxidative stress which is eliminated by the plant´s antioxidative apparatus accumulating various low-molecular weight compounds (e.g. ascorbate, glutathione, phytochelatins, etc.) and/or by activity of detoxification enzymes (catalases, peroxidases, superoxide dismutases, etc.) [15]. Arsenic disrupts vital cellular processes by binding to thiol groups and co-factors of enzymes (As^{3+}), or by replacing P in important biochemical reactions (As^{5+}). The binding of As^{3+} to thiols represents the key detoxification mechanism of both forms and results in the retention of a major part of As taken up by plants in roots [16]. Similar to many typical heavy metals like Cd or Pb, As impairs photosynthetic performance [21,22], but does not strongly affect genes related to carbon metabolism [15]. On the contrary, As causes dramatic changes in nitrogen transport and metabolism, altering the amino acid pools and energetic balance in cells [24]. There are few reports on how As affects different defense enzymes. Inhibitory but also activating effects have been reported for antioxidative enzymes [23, 25], chitinases in soybean, barley and maize [26], and for β-1,3-glucanases in maize and soybean [27]. The latter two enzymes re-modulate the cell wall composition, affect metabolite trafficking [28] and/or generate signaling molecules to trigger downstream defense cascades after a plant´s exposure to metal(loid) toxicity [21, 29], cold and drought [30,31]. Since several of them play pivotal roles in many morphological and developmental processes as well [32,33], they represent a good system to sensitively monitor the changes in both environmental conditions and developmental processes.

This work aims to reveal the character of changes in the previously-described plant responses to a broad range of N supply [3] with exposure to concurrent abiotic stress. In wheat plants exposed to sublethal dose of arsenic and NH_4NO_3 nutrition ranging between starvation to excess (toxicity), we studied the patterns

and activities of β-1,3-glucanase and chitinase enzymes. We aimed to i) reveal their sensitivity to arsenic stress, ii) evaluate the influence of N availability on the observed responses and iii) to estimate the effect of N supply on As uptake, plant vitality and tolerance. In contrast with most of the previously published studies, As was applied as As^{3+} and our discussion relates to this more toxic form.

Experimental Procedures

Plant Material, Cultivation and Experimental Design

Wheat (*Triticum aestivum* L. cv. Genoveva) was grown in a mineral nutrient solution [3]. Uniform seeds germinated on wet filter paper were transferred to constantly aerated plastic containers (15×15×20 cm) with 700 ml of basic Hoagland solution containing 0.2 mM NH_4NO_3, 5.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 2.0 mM $MgSO_4$, 0.5 mM Na_2SiO_3, 0.1 mM KH_2PO_4, 50 μM NaFe(III)EDTA, 50 μM H_3BO_3, 5 μM $MnCl_2$, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, and 0.1 μM Na_2MoO_3. In experimental variants, KNO_3 was fully replaced by K_2SO_4 and $Ca(NO_3)_2$ by $CaCl_2$ to modify the content of N (supplied solely in the form of NH_4NO_3) to the final total nitrogen concentrations of 0, 0.75 and 5.25 mM N (suboptimal concentrations), 7.5 mM N (optimal; comparable to the basic Hoagland solution), and 15, 25, 30 and 35 mM N (excess of N) [3]. On the basis of former trials, the solution was not exchanged. An additional set of the nitrogenous variants was supplemented with As^{3+} at a sublethal concentration of 5 mM, prepared from As_2O_3 [34]. The plants were grown in a climate-controlled chamber at 22ºC (6 a.m. to 6 p.m.) and 18ºC (6 p.m. to 6 a.m.) at a relative humidity of 60%, 16/8 h photoperiod and irradiance 300-400 μmol/m^2.s of PAR. Leaf and root tissue was sampled 10 days after cultivation in presence of As. Arsenic was removed from roots by washing with 0.5 mM EDTA twice for ~1 min and rinsing with distilled water. A set of plants was dried at 80ºC to constant weight to measure the dry weight (DW) of shoots and roots, and determine the content of elements (see below). For other analyses, the organs were frozen in liquid nitrogen and stored at -80ºC. The experiment was repeated 9 times independently in a randomized complete block design with one container with 15 plants (n = 135 plants per variant).

Tissue Content of Nutrients and Arsenic

Tissue organic nitrogen and phosphorus contents in leaf tips where analyzed following acid mineralization of the samples. Three experimental replicates per variant were quantified colorimetrically with an automatic FIAstar 5010 Analyzer (Tecator, Sweden); for details, see [35]. Since most of the nitrate taken up by roots is transported to the shoots in wheat, roots were not assayed [36]. Leaves and root samples were mineralized in a HNO_3 and H_2O_2 mixture (Anton Paar Multiwave 3000) and the As content was measured using ICP-MS (Perkin Elmer Elan 6000). For validation, certified reference materials NCS DC73349 and NCS DC73350 (China) were used.

Assays of Chitinases and Glucanases

Proteins were extracted from tissues as per [37]. A sample was taken as a pool of three individual plants per variant, and the enzyme analyses were performed in samples from three experimental replicates from each variant (n = 9 plants per variant). The activity of β-1,3-glucanases was determined with a 3,5-dinitrosalicylic acid (DNS) method [38]. The total chitinase activity in samples was measured fluorimetrically (Fluoroskan II microtiterplate reader, TITERTEK, Finland). Protein extracts (20 μl) were mixed with 30 μl of 300 μM 4-methylumbelliferyl-β-D-*N,N′,N′′*-triacetylchitotrioside in 0.1 M sodium citrate buffer (pH 3.0), and incubated at 37°C. Reactions were stopped by addition of 150 μl of 0.2 M Na_2CO_3 after 1 hour. Fluorescence was measured using excitation and emission filters 355 nm/450 nm and the enzyme activity was expressed as picomoles of methylumbelliferone generated per μg of soluble protein per hour.

For enzyme profile analyses, proteins (20 μg) were separated in 12.5% (w/v) polyacrylamide gels under standard conditions. The gels contained 0.01% (w/v) glycol chitin or 0.01% (w/v) laminarin (Sigma) as enzyme substrate. Enzyme profiles, profiles of acidic/neutral and basic/neutral isoforms (respectively) were detected after washing the gels in 50 mM sodium acetate buffer (pH 5.0) with 1% (v/v) Triton X-100 overnight. Chitinases were stained with 0.01% (w/v) Fluorescent Brightener 28 (Sigma) as described by [39]. The glucanase fractions were stained with 2,3,5-triphenyltetrazolium chloride (Sigma) [39]. As a measure of the enzyme activity, background-corrected integrated density (ID) of the bands was quantified using Scion Image software (http://www.scioncorp.com) [21]. The size of isoforms was determined as based on co-separated marker proteins after standard Coomassie-staining of gels. As isoforms separated only on the basis of their size can contain several isoforms of similar size, the conclusive value of these data might be limited; therefore, their patterns are not described in detail. Nevertheless, they were included in star icon plots to give a complete view on the extent of detected changes. Statistical evaluation of all the data is available in Supplement S1.

Statistical Analyses

One-way ANOVA and a Tukey post-hoc test, separately for shoots and roots, were performed to estimate the effects of nitrogen concentration in growth

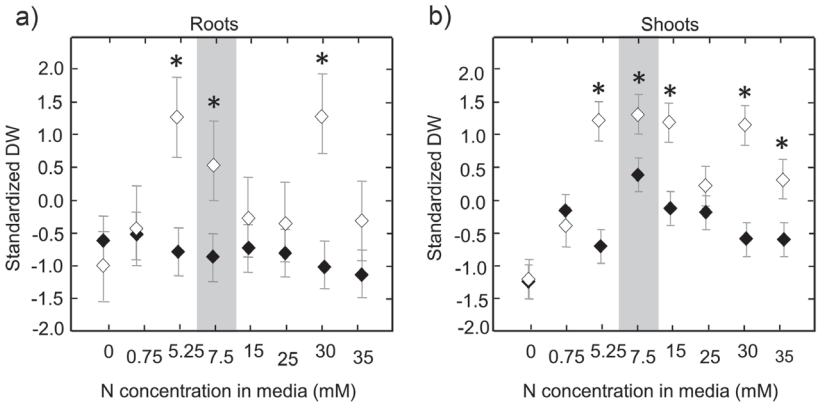

Fig. 1. Effect of 5 mM As^{3+} at different N concentrations in growth media on dry biomass of roots a), and shoots b) in absence (empty labels) or presence of As^{3+} (filled labels). Standardized average values of 5 plants per variant (n = 9 repetitions) with 95% confidential intervals are shown. Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$. Details on significance of N effects are given in Table S1.

media on the tested plant parameters. The relationship between the parameters was examined by a Spearman correlation coefficient. For clarity, the data for individual parameters were standardized (z) as $z = (x - \mu)/\sigma$, while x is the actual value of the parameter in a variant, μ is the average value of all variants and σ is the standard deviation for the given parameter. Standardized values of all parameters are presented as star icon plots that indicate the impact of N concentration. Each ray represents a different variable and its length represents the relative value of a particular variable. An overview of the statistical significance for the discussed parameters is given in Supplementary Table S1.

Results

Plant Growth

Nitrogen in media at both sub- and supra-optimal concentrations restricted plant growth and resulted in lower dry weights compared to optimal concentrations (Fig. 1a,b). An additional decrease of shoot dry mass was apparent in response to As in the media ($p<0.001$, $F = 54.51$) at 5.25, 15 and 30 mM N (Fig. 1b). The interaction between the effects of the two (stress) factors is significant ($p<0.001$, $F = 6.14$; Table S1). Similar effects were obvious for roots at 5.25 and 30 mM N as well (Fig. 1a).

Content of Elements

The relationship between N supply and its content in shoot tissue was convex, confirming the lowest N contents at limiting N concentrations. Arsenic toxicity disturbed this trend at concentrations ≤15 mM N and generally decreased the shoot N content (Fig. 2a). Exceptions were the increased N content in starving plants and the absence of any change at the optimal 7.5 mM N (Fig. 2a). The content of P in shoots, itself not dependent on nutrition up to 30 mM N [3], varied in response to As at ≥7.25 mM N in media (Fig. 2b).

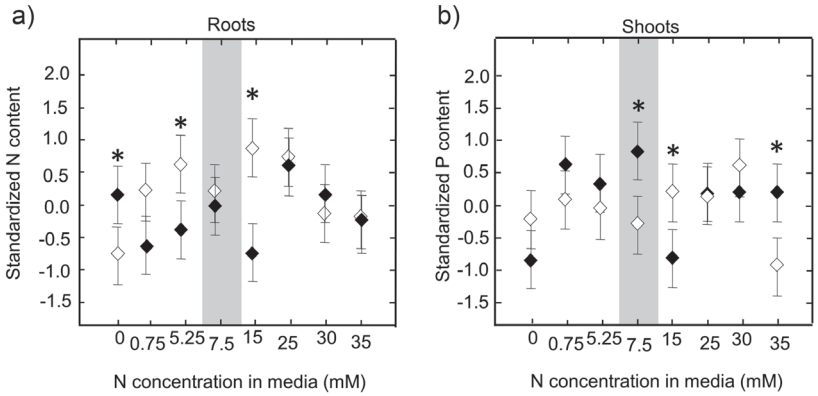

Fig. 2. Effect of 5 mM As^{3+} at different N concentrations in growth media on content of N a) and P b) in shoots when grown in absence (empty labels) or presence of As^{3+} (filled labels). Standardized average values with 95% confidential intervals (n = 3) are shown. Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$. Details on significance of N effects are given in the Table S1.

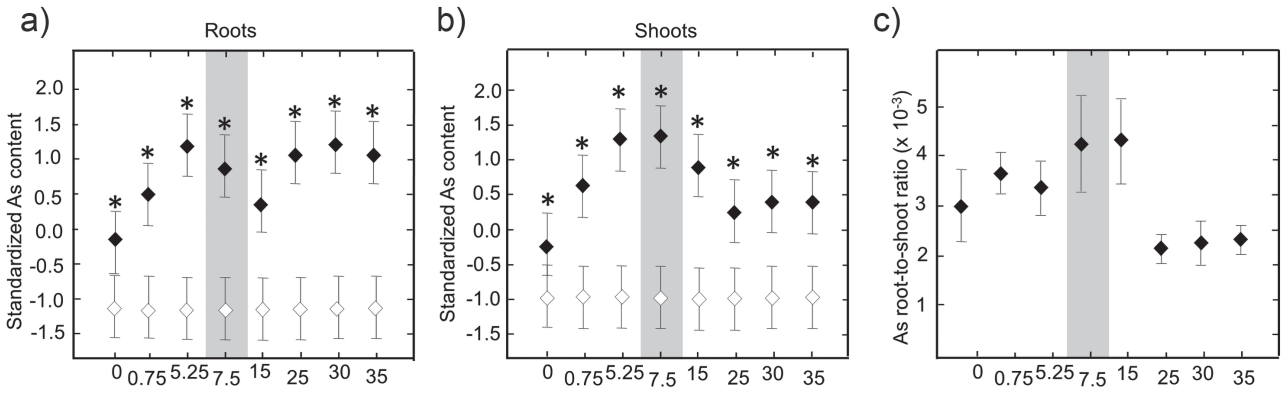

Fig. 3. Influence of 5 mM As^{3+} at different N concentrations in growth media on As content (g^{-1} DW) in roots a) and shoots b) and on root-to-shoot As content ratio c). Data show values in tissues grown in absence (empty labels) or presence of As^{3+} (filled labels). Standardized average values with 95% confidential intervals (n = 3) are shown. Asterisks indicate statistical significance for As effect in individual variants at $p<0.05$. Details on significance of N effects are given in the Table S1.

Elevated P levels were measured in shoots at 7.5 and 35 mM N, but less P was recorded at 15 mM N (Fig. 2b).

Arsenic accumulated predominantly in wheat roots. The accumulation rate was markedly affected by nitrogen availability in the range of 0-5.25 mM N (for both tissue types $p<0.05$; Fig. 3). Only 129.7±12.0 (SE) $\mu g.g^{-1}$ As was detected in poorly fed roots, in contrast to the highest As content of 350.8±17.2 $\mu g.g^{-1}$ recorded at 5.25 mM N (Table S2). At other optimal N concentrations, the root As content slightly dropped or remained at comparable levels at high N concentrations. A low amount of As was translocated to shoots. The highest average As content in shoots (1.28±0.61 $\mu g.g^{-1}$) was recorded at N concentrations close to the optimum (Fig. 3b). The shoot-to-root ratio of As content appeared lower at high N supply (Fig. 3c). However, neither the influence of N availability, As accumulation nor their interactive effects on the content of assayed elements was statistically significant for the complete set of experimental plants (Suppl. Table S1), indicating complexity of the underlying As uptake mechanisms.

Enzyme Activity of PR Proteins

A profound effect of N nutrition conditions on activity of PR proteins has been reported [3] and a possible impact on the defense mechanism under stress has been expected.

Chitinases

The overall activity of chitinases in wheat tissues markedly varied in response to As as dependent on N nutrition. In roots, activity levels were suppressed mostly at low as well as ≥15 mM N concentrations (Fig. 4a). Unlike in shoots, enhancement of enzyme activity was marked except for the two highest N concentrations (no effect or suppression; Fig. 4b).

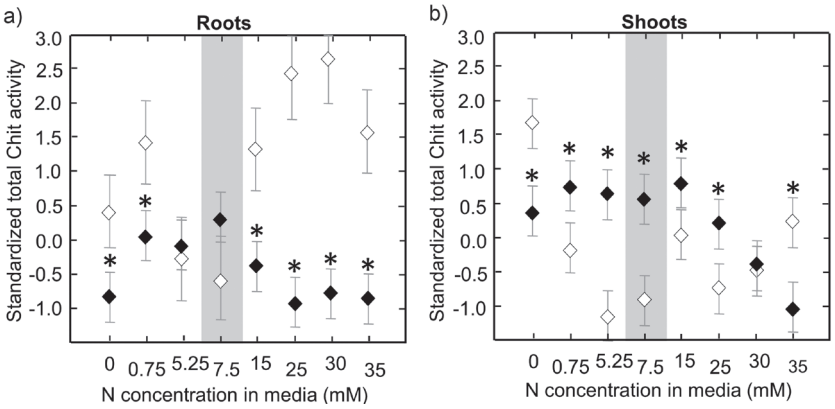

Fig. 4. Influence of 5 mM As^{3+} at different N concentrations in growth media on total activity of chitinases in roots a) and shoots b). Data indicate activity in absence (empty labels) or presence of As^{3+} (filled labels). Standardized average values with 95% confidential intervals (n = 3) are shown. Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$. Details on significance of N effects are given in the Table S1.

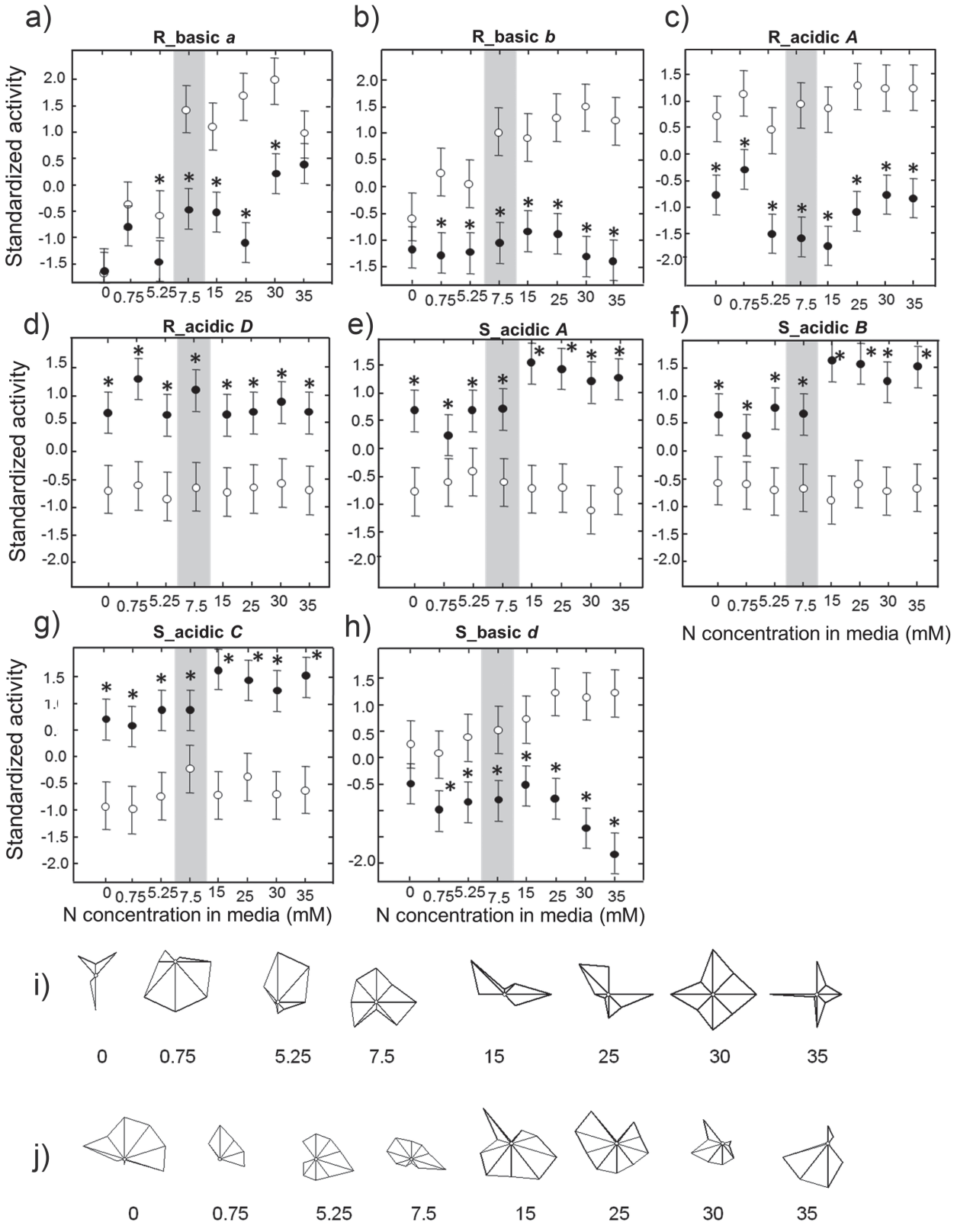

Fig. 5. Effect of 5 mM As^{3+} on activities of individual chitinases as dependent on N concentrations in growth media. The isoforms were detected in roots (a-d) and shoots (e-h) when grown in absence (empty labels) or presence of As^{3+} (filled labels). The star plots display the relative activity change of each quantified chitinase under stress, while rays correspond to individual isoform in roots (I, 8 items) and shoots (J, 10 items) at different N concentrations indicated below plots (in mM). In graphs, standardized average activity values with 95% confidential intervals (n=3) are shown. Different letters indicate significance at $P<0.05$. Chitinases given in star plots denote clockwise order from twelve o´clock position for 70, 50 kDa, basic *a-d*, acidic *A-D* (roots) and 65, 48, 40, 35 kDa, basic *a-d*, acidic *A-D* (shoots). Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$. Details on significance of N effects are given in the Table S1.

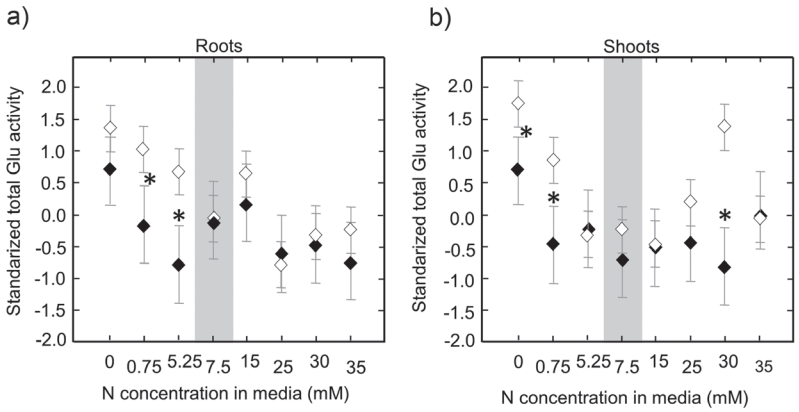

Fig. 6. Influence of 5 mM As^{3+} at different N concentrations in growth media on total activity of β-1,3-glucanases in roots a) and shoots b). Data indicate activity in absence (empty labels) or presence of As^{3+} (filled labels). Standardized average values with 95% confidential intervals (n = 3) are shown. Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$.

The interaction of As and nutrition on chitinases was significant only in shoots ($p<0.001$, $F = 6.21$).

For a more detailed analysis of chitinase enzymes in wheat tissues, we detected and quantified the activities of individual isoforms in separation gels. Two chitinase fractions detected in roots (70 and 50 kDa) comprised at least 4 acidic/neutral (*A-D*) and 4 basic/neutral (*a-d*) isoforms (Fig. S1), and half of them did respond to As. The acidic isoform *C* and the basic isoforms *a* and *b* appeared significantly inhibited by As (Fig. 5a-c), as opposed to the induced acidic isoform *D* (Fig. 5d). Some of these isoforms were significantly influenced by N conditions as well; the acidic *A* chitinase exerted relatively higher activities at both sub- and supra-optimal N concentrations, as opposed to the basic isoform *b* with generally low activities. The negative effect of As on the basic isoform *a* seems to be the strongest at optimal N but declines with better nutrition. The interaction of the As effect and the N supply was significant for the isoforms *C* and *b* (Suppl. Table S1).

Chitinases of four different sizes were detected in shoots (65, 48, 40 and 35 kDa), comprising at least 4 acidic/neutral (*A-D*) and 4 basic/neutral (*a-d*) isoforms (Fig. S1). Three acidic chitinases responded to As with significant induction, mainly at optimal or high N supply (Fig. 5e-g). Though we could not statistically support the effect of N for the whole plant set, the interactive effect of N with As was significant for isoforms *B* and *C* (Fig. 5f, g, Table S1). A single basic chitinase isoform (*d*) was inhibited by As (Fig. 5h), mainly at optimal or high N nutrition levels. Neither the effect of N at all concentrations nor the interactive effect with As were significant for this isoform (Table S1).

The icon plots of all quantified wheat chitinases (including those separated as based solely on size) summarize the relative activity values (responses) of individual isoforms, expressed as rays of corresponding sizes (Fig. 5i, j). The plots reflect variable responses of several chitinase isoforms to As toxicity, and suggest a considerable non-linear impact of N availability.

β-1,3-glucanases

The total activity of β-1,3-glucanases appeared to be suppressed by As in both organs (Fig. 6). This effect was observed at ≤5.25 mM N in roots ($p<0.001$, $F = 131.6$), and at both low and high N concentrations in shoots ($p<0.001$, $F = 34.0$). The combined effects of As and nutrition on these enzymes was, however, significant only in shoots ($p<0.001$, $F = 6.41$; Table S1). Up to four β-1,3-glucanases were detected in roots (150, 68, 45 and 35 kDa; Fig. S1; Fig. 7). Separation under native conditions revealed 4 acidic isoforms (*A-D*), of which only the *C* isoform was inhibited by As ($p<0.01$, $F = 18.8$) but only at ≤7.5 mM N (Fig. 7a). The interaction of this effect with N, however, was not significant (Table S1). On the other hand, both detected basic isoforms were activated by As at optimal nutrition or slightly higher N (isoform *a*), or with no obvious pattern regardless of the N supply (isoform *b*; Fig. 7b,c; Table S1). For the former basic isoform, we confirmed the effect of N supply ($p<0.01$, $F = 3.34$) as well as of its interaction with As ($p<0.001$, $F = 5.63$).

At least five β-1,3-glucanases of different sizes were present in shoots (150, 68, 50, 38 and 30 kDa), however, we detected only two acidic and a single basic isoform in native gels upon charge separation (Fig. S1; Fig. 7d,e). The acidic isoforms *A* and *B* were significantly induced in the presence of As and the former shows responsiveness to N conditions as well ($p<0.001$, $F = 6.99$). Importantly, the interactive effect of As and N was significant for both isoforms (Table S2) and the basic isoform was not induced by either studied factor. The icon plots of all quantified wheat β-1,3-glucanases (including those separated by size) suggest more decreased activities by As at limiting N concentrations in both organs. In shoots, the amplitudes of changes were highest at N concentrations between 15-30 mM N in media (Fig. 7 f,g).

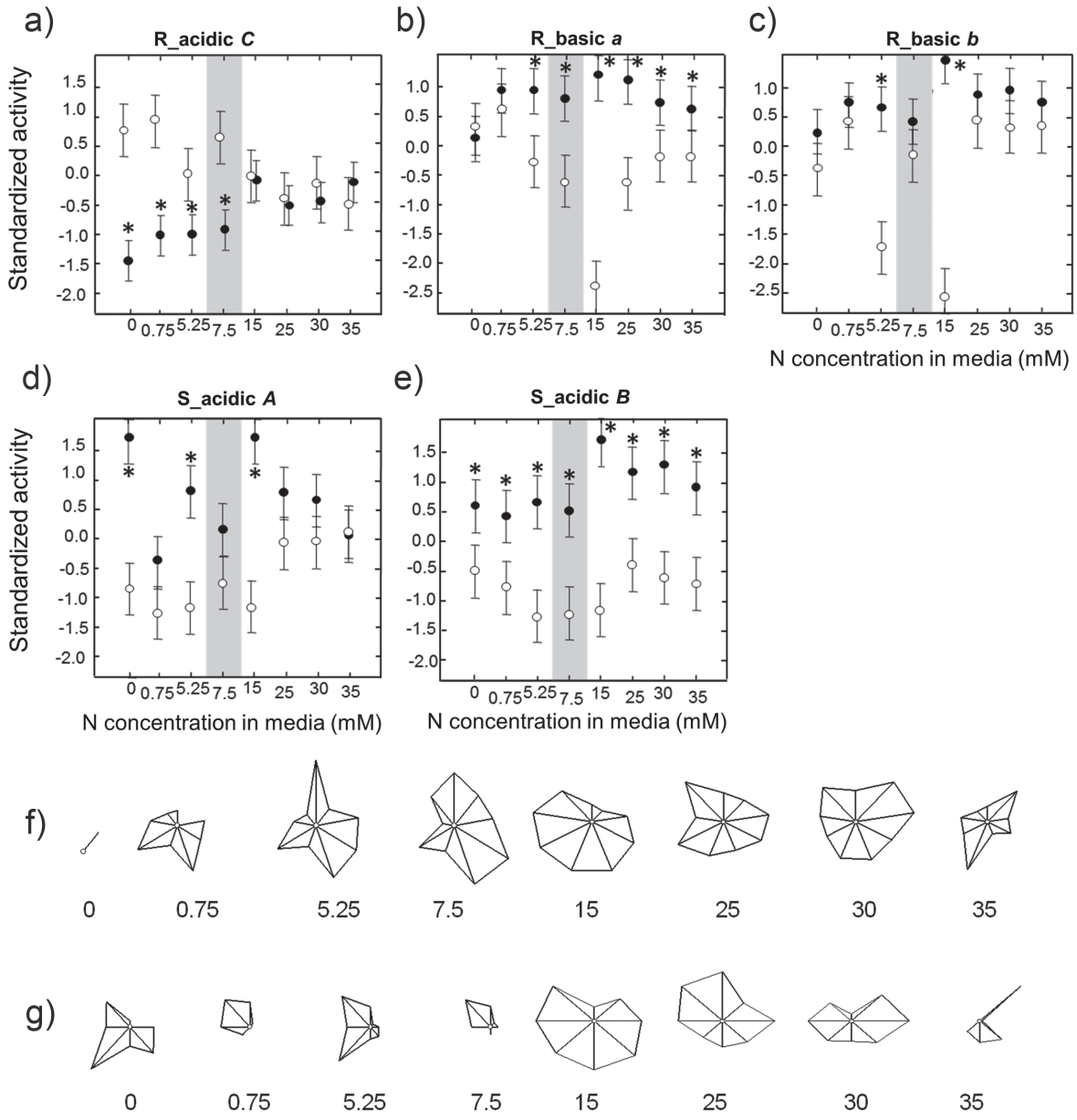

Fig. 7. Effect of 5 mM As^{3+} on activities of individual β-1,3-glucanases at different N concentrations in growth media. The isoforms were detected in roots (a-c) and shoots (d-e) when grown in the absence (empty labels) and presence of As^{3+} (filled labels). The star plots display the relative activity change of each quantified β-1,3-glucanases under stress, while rays correspond to individual isoforms in roots (f, 9 items) and shoots (g, 8 items) at different N concentrations indicated below plots (in mM). In graphs, standardized average activity values with 95% confidential intervals (n=3) are shown. Asterisks indicate a statistical significance for the As effect in individual variants at $p<0.05$. Beta-1,3-glucanases given in star plots denote clockwise order from twelve o´clock position for 150, 68, 45, 35 kDa, acidic *A-D,* basic *a-b* (roots) and 150, 68, 50, 38, 30 kDa, acidic *A-B,* basic *a* (shoots).

Discussion

Nitrogen nutrition positively coincides with plant amino acid content, but limits the metabolism of organic acids [1]. While both groups of metabolites comprise important defensive compounds, the former are believed to contribute more markedly to disease resistance, at least in relation to biotic stress [41]. Responses of different nitrogenous metabolites (e.g. proline or different enzymes) to stress thus logically depend on N availability, but the variability of experimental conditions in single studies, limited range of N concentrations, different forms of supplied N and other minor differences still generate a limited amount of comparable data to make reliable conclusions.

The effects of the eight different N concentrations on wheat growth, photosynthetic pigments and some of defense compounds have already been described, and the non-linear pattern of these responses has been emphasized [3]. Our present results showed that the presence of As^{3+} restricted the growth of experimental wheat plants similarly to some other species [41, 42],

Table 1. Correlation coefficient (*R*) of linear regression models between parameters at the presence of As^{3+} in media and corresponding statistical significance (p).

Parameter 1	Parameter 2	*R*	p
Shoots			
Basic β-1,3-glucanase *b*	N in media	0.88	0.004
Basic chitinase *d*	N in media	0.95	0.001
Roots			
Acidic chitinase *A*	N in media	0.83	0.010
Basic chitinase *a*	N in media	0.76	0.028
Basic chitinase *b*	N in media	0.93	0.001
Total β-1,3-glucanases	N in media	-0.83	0.010
Total β-1,3-glucanases	Total chitinases	-0.81	0.015

but more severely at optimal or high N concentrations (Fig. 1). No effect was observable at low nutrition. This heterogenous effect is likely the result of several factors that interfere with energy production/consumption. First, As^{3+} reduces photosynthetic rate e.g. by decreasing chlorophyll content [21, 22], inhibiting the enzymes responsible for their synthesis in plastids [43], replacing essential metallic ions such as Fe^{2+}, Zn^{2+} and Mg^{2+} [17] and by hampering ATP and NADPH syntheses [44]. Better N nutrition can counteract these impacts; N is not only one of main constituents of chlorophyll and Rubisco [45], but also contributes to the protection of the photosynthetic apparatus providing key players of antioxidation and detoxification such as glutathione (GSH) and phytochelatins (PCs) [46, 47]. Nevertheless, the collinearity of shoot dry weight with tissue N content was interrupted during the exposure to As^{3+} (Fig. 2a), confirming the direct interaction of As^{3+} with N uptake and metabolism [15].

Arsenic affects tissue nutrient contents as dependent on its chemical form and oxidation stage; unfortunately, reports dealing with As^{3+} are rather scarce as compared to As^{5+}. Similarly, there are fragmented pieces of knowledge on the potential effects of N nutrition and its forms on As^{3+} accumulation. Poorly-fed roots accumulated the lowest amount of As^{3+}, corroborating mild or absent impacts on biomass production. A similar effect of N-deficiency has been described for As^{5+} in rice roots but not shoots [48], and also for other heavy metals like Cd in barley [42] or chamomile [49]. Limited N supply mediated higher As^{3+} tolerance in algae, though the faster uptake rate indicated an increased amount of responsible transporters [50]; reports on the induced synthesis of such transporters under the low-N conditions are, however, still missing. More importantly, as mentioned above, poor nutrition might limit the potential targets of As^{3+} toxicity such as SH-containing proteins and low molecular weight thiols [51]. Their intracellular levels can further decrease through the inhibition of nitrate and nitrite reductases [52], which regulate the assimilation of nitrate and are involved in protein synthesis. The latter scenario is probably of a lesser importance at high N supply as the N content in wheat shoots remained unaffected in the presence of As (Fig. 2).

At N concentration ≥25 mM, the amount of accumulated As^{3+} was high in roots but very low in shoots. Possible reasons could be a drop of pH [53], but in the present experiment it remained unchanged (data not shown). We also exclude a "dilution" of As due to a relatively higher biomass [54]. Root-to-shoot translocation of As species is mostly limited and varies among plant species [55], but apparently alters with N supply. Restricted accumulation of As^{5+} has also been observed in rice shoots during the growth in a soil supplemented with 15 mM N [56]. On the contrary, arsenic alters the uptake and metabolism of other elements in plants. Interference with P uptake and metabolism is more often discussed for As^{5+} because of structural similarity with phosphate. Our results revealed that As^{3+} differently affected tissue P content in wheat shoots under different conditions of N supply as a result of several, yet poorly understood and possibly counteracting mechanisms. Comparable As^{3+} concentrations were reported to inhibit P uptake [57], while in some species As levels of 50 mg/l (0.67 mM) were also efficient [58]. Moreover, interrupted water balance, which usually occurs in plant tissues under As stress, decouples the P and N cycles [59].

The available nutrient resources for each plant are considered limited, and there exists a trade-off between investments (directing resources) to growth and defense [60]. A surplus of N has been shown to alleviate the toxicity of As^{5+}, possibly by fuelling and boosting the antioxidative apparatus [48, 56]. Though the restrained As^{3+} transfer in well-nourished wheat plants could be considered a sign of tolerance, the associated drop of biomass does question the benefit of high surplus N on plant vitality. Especially at the most excessive N concentrations, N toxicity likely additively contributes to detrimental effects of As^{3+}, e.g. on the contents of some pigments and the growth [22]. Our data on overall activity of chitinases in wheat confirm this; while N deficiency alone might be responsible (at least partly) for relatively low activity values in roots under both normal and As stress conditions [61], excess of N apparently strengthened the impact of As toxicity on chitinases and caused suppression of overall activity (Fig. 4a). In shoots, on the other hand, As^{3+} mostly induces chitinase activity, but this effect mitigates with increasing N and finally drops to the relatively lowest values (Fig. 4b). An analogous pattern fits for the total activity of β-1,3-glucanases at N shortage in shoots. These patterns, however, mask the activity of few isoforms with opposed behaviour; e.g. several acidic chitinases in shoots exert activation by increasing N

supply (Fig. 5). Nevertheless, the generally accepted positive correlation between N nutrition, activity of PR proteins and final resistance [60] apparently should be re-considered or at least handled more carefully since it applies for a few isoforms only (Table 1). Star plots on responses of individual enzyme isoforms show an obvious suppression of several chitinase and β-1,3-glucanase members by high N in roots at the same time point (Figs. 5i,j and 7f,g).

Though the studied enzymes have been widely researched in the context of biotic stress, their role during abiotic stress including tolerance to heavy metals and As^{3+} remains unclear. Recently, several chitinase and β-1,3-glucanase transcripts were induced and a few were repressed in rice roots exposed to As^{3+}, while their role in cell-wall biogenesis and/or organisation under metal stress has been proposed [62]. Rapid activation of chitinases has been associated with tolerant genotypes [29], possibly by generating the signals for the activation of downstream responses. For β-3-glucanases, growing amounts of evidence suggests a regulatory role in plasmodesmal permeability and metabolite trafficking under metal stress via the breakdown of callose [28, 63]. Regardless of their role, in our study, some of these enzymes were responsive to both As^{3+} and N supply, confirming the cross-talk between availability/allocation of resources and stress responses [64]. Direct evidence of the activation of these enzymes by N supply has been reported only for a β-1,3-glucanase in cotton, where it resulted in the highest fiber strength at optimal, but not at lower or higher N supply, by hydrolysing β-1,3-glucan and providing UDP-glucose for cellulose synthesis [65]. The effects on plant tolerance were not tested in the last-mentioned study, but fiber enforcement has been associated with decreased (biotic) stress tolerance [66]. Responsivity of some PR proteins to N supply has been demonstrated also in wheat [3], rice [62] and poplar [13].

Conclusion

As^{3+} ions suppressed wheat growth and altered metabolism of both N and P in shoots. On the other hand, N nutrition supply affected As^{3+} accumulation and transfer to shoots. Though higher N supply appears to protect shoots from As toxicity, plant growth and physiological processes are apparently compromised. Responses of studied PR enzymes suggest counterbalancing of actual limitations caused by N availability and As stress, while the interactive effect of both factors proved significant for several enzyme isoforms. Regardless of the exact biological function of these enzymes, their responses contradict the generally accepted view on the activation of PRs as a result of higher nutrition. At the same time, an optimal N nutrition needs to be more carefully defined, especially under the conditions of (multiple) stress. Studying the effects of different fertilization regimes on the activity of (defense) enzymes under multiple stresses might bring knowledge for the efficient fertilization and food safety strategies.

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Conflict of Interest

The authors report no conflict of interest.

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Supplementary Material

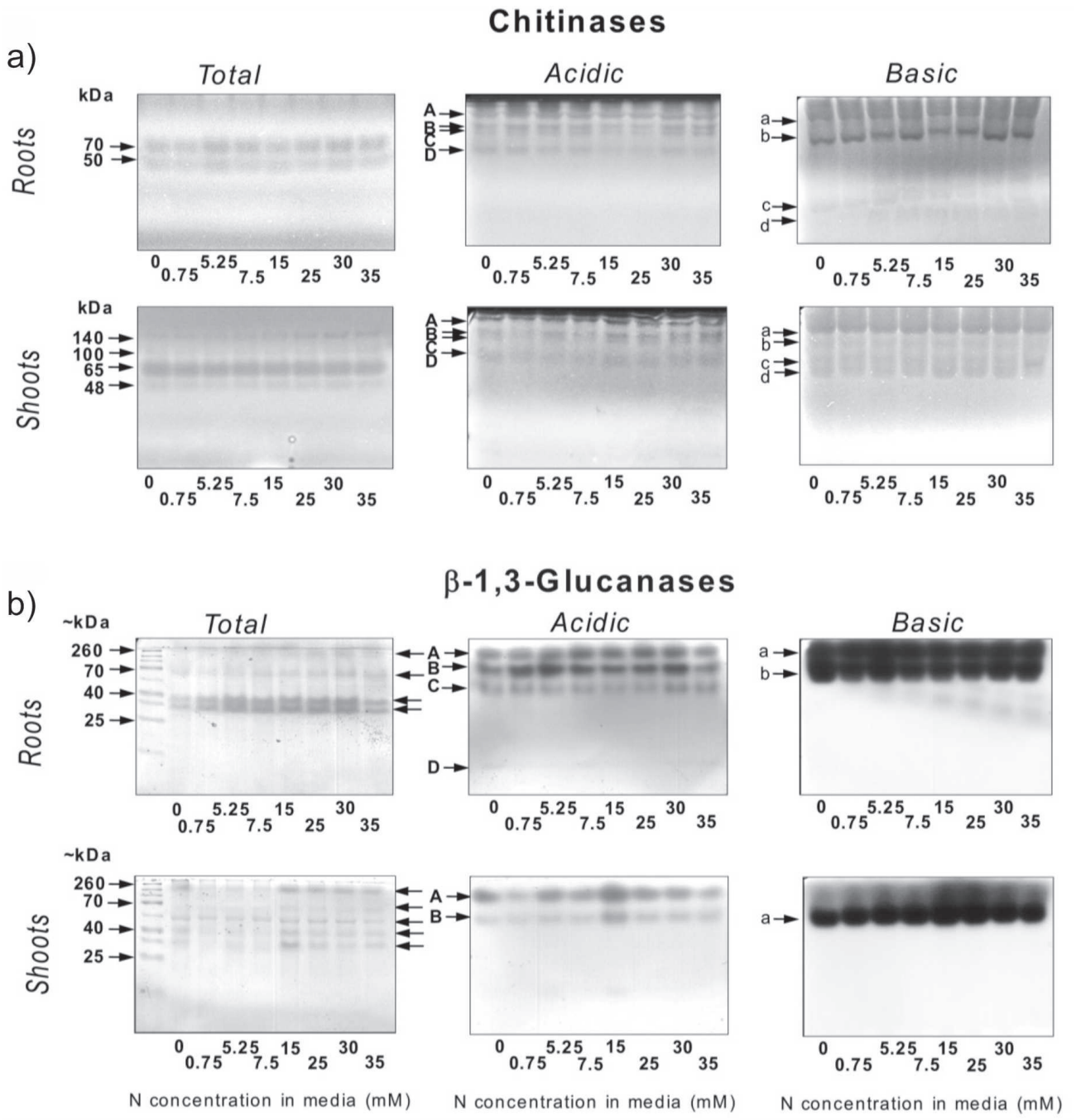

Fig. S1. Isoforms of chitinases and β-1,3-glucanases from wheat roots and shoots grown at 5 mM As^{3+} and different concentrations of NH_4NO_3 in media (given below pictures, within 0-35 mM N). Reference samples were from plants without As (not shown). Detection in polyacrylamide gels was performed using specific enzyme substrates upon semi-denaturing conditions (separation of all isoforms by size; Total) or under native conditions (acidic/neutral or basic/neutral isoforms were separated). Size of enzyme fractions was determined on the basis of a co-separated molecular size marker. After scanning, the band intensities were determined using ScionImage software and the obtained values were statistically analysed for effect of As^{3+}, N supply and both factors.

Table S1. Overview of statistics on the influence of As^{3+}, nitrogen supply and their interaction in wheat shoots and roots.

Variable	AsIII stress			N concentration			Interaction of stress x concentration		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Growth parameter									
DW_root	52,13	1	<0,001	5,40	7	0,015	8,11	7	0,004
DW_shoot	2,13	1	0,148	57,13	7	<0,001	60,53	7	<0,001
Content of elements									
N content_shoot	0,09	1	0,768	0,67	7	0,699	1,57	7	0,193
P content_shoot	1,41	1	0,246	0,69	7	0,682	0,87	7	0,542
As content_shoot	54,64	1	<0,001	0,85	7	0,561	0,82	7	0,577
As content_root	48,93	1	<0,001	0,38	7	0,890	0,38	7	0,887
Total enzyme activities									
Chitinases_root	28,16	1	<0,001	1,28	7	0,364	3,41	7	0,054
Chitinases_shoot	10,52	1	0,002	6,65	7	<0,001	6,21	7	<0,001
β-1,3-Glucanase_root	377,29	1	<0,001	195,37	7	0,000	49,67	7	<0,001
β-1,3-Glucanase_shoot	28,53	1	<0,001	6,57	7	<0,001	3,91	7	0,001
Activities of chitinase isoforms									
Chitinase_70 kDa_root	12031,32	1	<0,001	12,26	7	0,001	12,29	7	0,001
Chitinase_50 kDa_root	38614,13	1	<0,001	30,32	7	<0,001	38,41	7	<0,001
Acid chitinase_*A*_root	28,83	1	0,001	1,38	7	0,328	0,32	7	0,922
Acid chitinase_*B*_root	29,55	1	0,001	0,62	7	0,731	0,06	7	0,999
Acid chitinase_*C*_root	90,92	1	<0,001	1,34	7	0,343	0,57	7	0,761
Acid chitinase_*D*_root	166,45	1	<0,001	1,18	7	0,407	0,32	7	0,926
Basic chitinase_*a*-root	24,08	1	0,001	7,52	7	0,005	1,67	7	0,244
Basic chitinase_*b*-root	374,38	1	<0,001	8,00	7	0,004	7,78	7	0,005
Chitinase_65 kDa_shoot	28411,00	1	<0,001	34,40	7	<0,001	48,89	7	<0,001
Chitinase_48 kDa_shoot	2206,20	1	<0,001	3,75	7	0,013	1,37	7	0,285
Chitinase_40 kDa_shoot	2472,00	1	<0,001	1,73	7	0,173	2,41	7	0,069
Chitinase_35 kDa_shoot	1053,77	1	<0,001	0,63	7	0,724	2,83	7	0,084
Acid chitinase_*A*_shoot	123,47	1	<0,001	0,73	7	0,645	1,71	7	0,135
Acid chitinase_B_shoot	297,09	1	<0,001	1,94	7	0,130	5,25	7	0,003
Acid chitinase_C_shoot	284,16	1	<0,001	2,00	7	0,119	3,66	7	0,015
Acid chitinase_D_shoot	32,00	1	<0,001	0,15	7	0,989	0,36	7	0,901
Basic chitinase_*a*_shoot	2,44	1	0,128	1,37	7	0,250	2,19	7	0,062
Basic chitinase_*b*_shoot	27,10	1	0,001	0,84	7	0,587	0,91	7	0,541
Activities of β-1,3-glucanase isoforms									
β-1,3-Glucanase_150 kDa_root	101,17	1	<0,001	0,15	7	0,988	0,26	7	0,956
β-1,3-Glucanase_68 kDa_root	125,13	1	<0,001	5,16	7	0,017	7,17	7	0,006
β-1,3-Glucanase_45 kDa_root	100,51	1	<0,001	4,30	7	0,029	3,15	7	0,065
β-1,3-Glucanase_35 kDa_root	409,73	1	<0,001	4,12	7	0,032	5,70	7	0,013
Acid β-1,3-glucanase_*A*_root	1,53	1	0,252	0,77	7	0,626	0,44	7	0,851

Table S1. Continued.

Acid β-1,3-glucanase_*B*_root	0,63	1	0,452	1,65	7	0,250	0,64	7	0,715
Acid β-1,3-glucanase_*C*_root	18,79	1	0,002	0,38	7	0,893	3,01	7	0,073
Basic β-1,3-glucanase_*a*_root	91,98	1	<0,001	4,51	7	0,025	10,32	7	0,002
Basic β-1,3-glucanase_*b*_root	42,28	1	<0,001	3,93	7	0,037	6,57	7	0,008
β-1,3-Glucanase_150 kDa_shoot	0,69	1	0,415	6,52	7	<0,001	8,15	7	<0,001
β-1,3-Glucanase_68 kDa_shoot	1,96	1	0,180	1,27	7	0,327	0,44	7	0,861
β-1,3-Glucanase_50 kDa_shoot	1098,58	1	<0,001	2,23	7	0,088	1,29	7	0,317
β-1,3-Glucanase_38 kDa_shoot	56,71	1	<0,001	1,04	7	0,474	0,90	7	0,550
β-1,3-Glucanase_30 kDa_shoot	20,33	1	0,002	8,26	7	0,004	10,67	7	0,002
Acid β-1,3-glucanase_*A*_shoot	119,45	1	<0,001	6,69	7	<0,001	8,90	7	<0,001
Acid β-1,3-glucanase_*A*_shoot	177,45	1	<0,001	1,76	7	0,165	4,08	7	0,009
Basic β-1,3-glucanase_*a*_shoot	7,52	1	0,010	2,78	7	0,022	0,69	7	0,677

F - F value
df- degree of freedom
P - significance

Table S2. Data of various parameters in wheat roots (R) and shoots (S) when grown in presence (+) or absence (–) of 5 mM As^{3+}. Different letters indicate significant difference between treatments at $P<0.05$

Growth			0 mM N	0.75 mM N	5.25 mM N	7.5 mM N	15 mM N	25 mM N	30 mM N	35 mM N
Dry weight (mg)	R	– As	1.47±0. 12 ab	1.62±0. 18 ab	2.63±0. 11 c	2.92±0. 18 bc	2.26±0. 23 abc	1.99±0. 17 abc	3.11±0.23 c	2.11±0. 22 abc
	R	+ As	1.11±0. 08 ab	1.10±0. 07 abc	1.01±0. 05 ab	1.00±0. 07 ab	1.02±0.05 ab	0.97±0.04 ab	0.93±0. 06 a	0. 74±0.05 a
Dry weight (mg)	S	– As	8.14±0.31 a	9.84±0. 0.30 abc	13.33±0. 40 de	13.86±0. 32 e	13.42±0. 23 de	11.18±0. 31 bcd	13.11±0. 33 de	11.49±0.41 cde
	S	+ As	8.10±0. 31 a	10.58±0. 35 bc	9.28±0. 50 ab	11.83±0. 67 bc	10.67±0. 62 bc	10.55±0.52 bc	9.49±0. 57 ab	9.52±0. 55 ab
Content of elements										
N content (mg/g)	S	– As	4. 06±0. 07 a	4. 53±0. 20 b	4. 67±0. 20 bc	4. 51±0. 11 b	4. 85±0. 06 c	4. 78±0. 32 c	4. 41±0. 05 ab	4. 35±0. 15 b
	S	+ As	4. 54±0. 24 bc	4. 09±0. 23 ab	4. 10±0. 14 a	4. 54±0. 15 ab	3. 71±0. 08 a	4. 66±0. 37 c	4. 42±0. 26 bc	4. 30±0. 12 a
P content (mg/g)	S	– As	0. 75±0. 04 ab	0. 76±0. 06 ab	0. 76±0. 04 ab	0. 73±0. 05 ab	0. 78±0. 02 b	0. 77±0. 02 ab	0. 81±0. 01 b	0. 67±0. 07 b
	S	+ As	0. 64±0. 05 a	0. 77±0. 02 bc	0. 78±0. 03 bc	0. 84±0. 05 c	0. 64±0. 04 a	0. 8±0. 07 b	0. 78±0. 04 b	0. 81±0. 02 b
As content (mg/g)	R	– As	0. 66±0. 05 a	0. 74± 0. 02 a	0. 20±0. 01 a	0. 21±0. 02 a	0. 19±0. 02 a	0. 18±0. 01 a	0. 16±0. 01 a	0. 17±0. 03 a
	R	+ As	129. 67±9. 87 a	237. 60±28. 51 ab	350. 83±14. 06 b	294. 33±9. 98 ab	272. 77±61. 50 ab	307. 7±25. 13 b	334. 43±55. 74 b	321. 17±19. 68 b
As content (mg/g)	S	– As	0. 01±0.00 a	0. 01±0.00 a	0. 02±0.00 a	0. 02±0.00 a	0. 02±0.00 a	0. 01±0.00 a	0. 01±0.00 a	0. 01±0.00 a
	S	+ As	0. 39±0. 06 a	0. 88±0. 02 bc	1. 26±0. 24 bc	1. 28±0. 50 bc	1. 07±0. 10 b	0. 67±0. 05 ab	0. 77±0. 17 ab	0. 76±0. 06 ab
Enzyme activity - β-glucanases (Glu)*										
Total Glu activity	R	– As	122. 30± 4. 26 f	110. 46±2. 67 h	110. 25±18. 08 gh	84. 79±1. 74 ef	116. 67±9. 94 g	80. 11±9. 58 ab	75. 39± 4. 06 cde	79. 35±4. 80 cdef
	R	+ As	84. 67±28. 22 gh	65. 74±21. 91 def	54. 33±18. 11 ab	62. 53±20. 84 ef	49. 62±16. 54 f	37. 57±12. 52 abcd	41. 61±13. 87 bcd	40. 33±13. 44 a
Total Glu activity	S	– As	123. 56±19. 93 d	97. 37±6. 45 cd	57. 02±2. 85 ab	61. 94±4. 10 abc	53. 69±5. 93 ab	73. 90±4. 45 abc	115. 10±13. 80 d	67. 27±5. 77 abc
	S	+ As	86. 52± 4. 01 bcd	56. 01±2. 19 ab	60.00±2. 07 abc	49. 80±3. 04 a	55. 53±4. 13 ab	49. 51±3. 18 ab	44. 09± 4. 11 a	69. 87±2. 04 abc
Glu-150 kDa	R	– As	33. 76±0. 57 a	37. 08±0. 12 a	34. 71±0. 45 a	35. 60±0. 18 a	34. 59±0. 67 a	34. 18±0. 61 a	36. 22±0. 41 a	33. 29±1. 16 a
	R	+ As	49. 58±1. 26 a	51. 42±1. 15 a	54. 02±1. 62 ab	54. 29±0. 54 ab	51. 33±0. 97 ab	52. 11±1. 56 ab	52. 84±2. 19 ab	52. 87±1. 60 ab
Glu-68 kDa	R	– As	43. 71±0. 98 bcde	29. 57±0. 41 abcd	28. 7±0. 66 ab	38. 46±0. 08 bcd	29. 26±0. 85 abc	25. 98±2. 01 abcd	30. 40±0. 63 ab	23. 16±1. 04 a
	R	+ As	43. 19±1. 95 bcde	38. 90±0. 86 bcde	41. 47±0. 69 bcde	42. 75±0. 15 de	41. 36±1. 22 bcde	41. 57±0. 92 cde	45. 10±0. 51 e	43. 20±1. 10 e
Glu-45 kDa	R	– As	110. 36±2. 06	128. 60±1. 49	119. 81±2. 76	116. 90±0. 45	107. 82±1. 80	113. 15±0. 68	119. 80±1. 19	115. 91±1. 82
	R	+ As	97. 09±0. 08	103. 59±1. 54	105. 69±1. 56	106. 04±0. 23	106. 48±1. 10	107. 45±1. 11	109. 61±1. 44	102. 44±0. 48
Glu-35 kDa	R	– As	126. 72±1. 70 abcde	141. 29±1. 33 e	135. 81±3. 3 bcde	132. 29±0. 49 cde	121. 34±1. 94 abcde	131. 99±2. 27 bcde	134. 57±0. 49 de	132. 64±10 de
	R	+ As	102. 35±0. 43 a	106. 07±1. 76 abc	106. 1±1. 27 abcd	110. 48±0. 14 abcd	113. 49±0. 56 abcde	114. 58±1. 07 abcd	113. 07±1. 18 bcde	106. 9±0. 59 ab

Table S2. Continued.

Glu-150 kDa	S	– As	37. 88±0. 60 ab	29. 61±0. 32 ab	27. 07±0. 41 a	30. 99±0. 12 ab	26. 80±0. 64 a	27. 83±0. 12 a	27. 82±0. 25 a	27. 36±0. 54 a
	S	+ As	29. 23±0. 83 ab	29. 32±0. 69 ab	29. 96±0. 21 ab	29. 92±0. 52 ab	29. 99±0. 78 ab	33. 99±0. 97 bc	28. 21±0. 41 a	28. 31±0. 48 a
Glu-68 kDa	S	– As	32. 27±0. 80	34. 27±0. 20	31. 94±0. 40	32. 78±0. 60	33. 72±0. 14	33. 43±0. 80	32. 78±0. 97	34. 68±0. 41
	S	+ As	32. 99±0. 42	33. 93±0. 44	32. 89±0. 49	32. 78±0. 22	34. 98±0. 57	34. 21±0. 32	35. 68±0. 35	34. 14±1. 18
Glu-50 kDa	S	– As	47. 02±0. 51 c	46. 47±0. 11 cd	48. 34±0. 57 c	48. 16±0. 39 c	46. 81±0. 20 c	47. 68±0. 15 c	49. 05±0. 37 c	46. 94±0. 97 c
	S	+ As	33. 51±0. 26 ab	33. 71±0. 49 ab	32. 97±0. 17 ab	33. 57±0. 27 ab	35. 19±0. 14 ab	34. 99±0. 47 bc	35. 31±0. 37 a	32. 08±0. 81 a
Glu-38 kDa	S	– As	57. 98±5. 42 f	45. 25±0. 73 e	42. 66±0. 22 de	43. 66±0. 08 de	40. 76±1. 48 bcde	42. 26±0. 93 cde	43. 6±0. 76 de	43. 73±0. 90 de
	S	+ As	33. 98±0. 58 abcde	28. 90±0. 36 a	31. 19±0. 59 ab	28. 95±0. 43 a	37±0. 25 abcde	33. 93±0. 58 abcd	33. 76±1. 58 abcd	32. 55±0. 97 abc
Glu-30 kDa	S	– As	71. 47±0. 10 e	36. 14±0. 56 bcd	32. 19±0. 76 abcd	33. 15±0. 79 abcd	28. 36±1. 50 abc	35. 36±2. 28 bcd	35. 92±2. 36 bcd	36. 32±2. 30 cd
	S	+ As	28. 86±1. 03 abc	22. 87±0. 87 a	28. 24±0. 09 abc	25. 81±0. 40 ab	41. 74±1. 38 d	33. 22±0. 96 bcd	30. 09±1. 92 abc	30. 06±1. 43 abc
AcGlu-A	R	– As	95. 62±4. 87	107. 05±1. 74	86. 99±0. 08	101. 74±0. 14	104. 34±0. 70	103. 36±4. 99	99. 82±10. 12	79. 01±2. 37
	R	+ As	67. 33±4. 23	81. 42±9. 10	90. 27±3. 67	96. 48±1. 38	92. 78±2. 14	89. 24±5. 12	89. 07±3. 77	82. 76±8. 12
AcGlu-B	R	– As	85. 53±8. 58 ab	116. 63±1. 34 b	102. 07±2. 50 ab	113. 36±5. 10 b	83. 12±3. 79 a	90. 72±2. 14 ab	93. 88±4. 83 ab	84. 45± 2. 13 a
	R	+ As	55. 97±0. 88 c	93. 73±2. 33 c	97. 90±10. 17 cd	105. 76±1. 28 c	108. 92±8. 67 abc	88. 13±5. 59 c	96. 92±8. 04 abc	77. 35±10. 18 cd
AcGlu-C	R	– As	77. 71±2. 56	92. 81±6. 50	72. 54±3. 87	78. 41±0. 37	70. 67±0. 78	68. 77±2. 53	73. 06±0. 98	71. 48±1. 64
	R	+ As	59. 31±1. 45	62. 86±0. 69	63. 01±0. 95	66. 37±2. 34	68. 70±3. 36	68. 15±0. 66	67. 27±3. 48	71. 71±0. 72
AcGlu-A	S	– As	38. 88± 4. 71 ab	31. 33±3. 44 a	33. 67±2. 32 ab	40. 17±0. 64 ab	33. 73±2. 51 ab	54. 55±2. 85 bc	53. 92±0. 72 bc	55. 70±1. 03 bc
	S	+ As	83. 67±48. 31 d	47. 45±27. 40 abc	68. 87±39. 76 cd	55. 48±32. 03 bc	83. 47±48. 19 d	68. 29±39. 42 cd	65. 55±37. 84 cd	54. 60±31. 52 bc
AcGlu-B	S	– As	81. 15±0. 91 abc	78. 31±0. 59 a	70. 39±2. 05 a	71. 36±0. 11 a	71. 78±1. 47 a	82.00±1. 93 abc	79. 64±1. 88 ab	78. 87±1. 95 ab
	S	+ As	95. 33±1. 66 cd	93. 90±1. 02 bcd	96. 14±2. 02 cd	94. 97±1. 60 cd	112. 24±2. 06 e	102. 52±1. 88 de	103. 53±3. 04 de	100. 69±3. 92 de
BasicGlu-a	R	– As	208. 20±5. 09 c	216. 79±3. 40 cd	207±1. 25 b	208. 29±1. 77 c	180. 44±2. 17 a	193. 95±5. 59 cd	204. 67±3. 26 cd	201. 65±6. 95 cd
	R	+ As	217. 22±2. 23 cd	227. 88±1. 09 cd	230. 35±1. 43 cd	228. 36±1. 01 cd	231. 14±2. 14 e	228. 86±2. 45 cd	226. 02±2.05 cd	224. 2±2. 82 cd
BasicGlu-b	R	– As	211. 06±0. 52	214. 38±2. 54	202. 32±1. 19	210. 29±1. 35	199. 25±3. 56	210. 79±3. 21	210. 68±30.11	208. 28±4. 99
	R	+ As	219. 47±2. 58	221. 04±0. 49	222. 87±2. 06	218. 86±0. 12	223. 77±2. 91	220. 11±1. 51	222. 53±1. 66	221. 46±2. 40
BasicGlu-a	S	– As	225. 84±2. 13	231. 25±1. 85	232. 8±2. 26	233. 43±1. 15	233. 42±0. 47	235. 41±1. 06	235. 01±2. 58	234. 74±3. 12
	S	+ As	227. 48±0. 34	229. 08±0. 22	229. 44±0. 47	229. 87±0. 55	232. 23±0. 39	233. 03±0. 94	230. 91±1. 41	228. 6±2. 07

Table S2. Continued.

Enzyme activity - chitinases (Chit)*										
Total Chit activity	R	– As	6925. 96±335. 68 b	11223. 78±1752. 86 bc	8800. 96±1411. 10 ab	8777. 49±2176. 62 a	8693. 12±1436. 19 bc	10043. 96±2001. 30 bcd	11824. 33±489. 31 bcd	14394. 4±414. 67 bcd
	R	+ As	4680. 59±191. 29 a	7955. 53±720. 71 bc	4519. 1±1824. 19 b	7385. 57±1383. 50 c	5032. 38±524. 64 ab	4150. 93±629. 38 a	3358. 52±353. 62 a	3581. 06±66. 30 a
Total Chit activity	S	– As	5227. 18±277. 56 d	3134. 96±226. 17 abc	1973. 77±53. 92 a	2080. 46±135. 86 a	3346. 5±99. 92 ab	2418. 60±78. 70 abc	2648. 26±145. 29 abc	3009. 27±162. 23 abc
	S	+ As	3763. 37±31. 78 abcd	4187. 07±147. 46 bcd	4089. 65±191. 93 bcd	3904. 61±242. 02 bcd	4261. 09±183. 02 cd	3589. 16±195. 24 abcd	2676. 01±98. 22 abc	2064. 58±218. 26 a
Chit-70 kDa	R	– As	117.47±2.68 d	121.09±1.46 bcd	117.56±0.28 abc	116.88±0.30 a	117.29±1.11 ab	119.46±0.77 cd	119.63±0.81 d	116.81±1.60 abcd
	R	+ As	174.08±0.75 e	173.92±0.96 e	174.02±0.35 e	173.79±0.50 e	171.88±0.69 e	172.93±0.45 e	175±0.62 e	176.75±1.67 e
Chit-50 kDa	R	– As	114.66±1.79 cd	118.53±1.48 d	115.21±1.25 ab	113.8±0.85 a	112.48±1.53 bc	114.6±0.84 bc	115.76±0.45 c	112.44±1.67 c
	R	+ As	157.59±0.85 ef	157.28±1.23 ef	155.47±0.69 f	155.55±0.99 ef	153.85±0.42 ef	154.34±0.54 e	157.12±0.76 ef	159.02±1.51 ef
Chit-65 kDa	S	– As	104.93±0.23 b	104.92±0.35 b	99.73±0.64 a	98.33±0.87 a	105.98±0.98 b	107.67±1.11 b	114.29±1.06 c	116.42±1.40 c
	S	+ As	167.58±0.49 e	167.34±0.49 de	166.48±0.41 de	165.62±0.40 de	164.84±0.47 de	164.73±0.42 d	164.6±0.27 de	165.13±0.18 de
Chit-48 kDa	S	– As	128.83±2.47 b	122.94±1.25 a	122.44±0.64 a	124.42±0.15 ab	125.19±0.25 ab	126.73±0.13 ab	124.35±0.66 ab	124.35±0.71 ab
	S	+ As	158.54±0.20 c	157.31±0.49 c	157.06±0.21 c	156.04±0.16 c	156.19±0.24 c	156.81±0.06 c	154.72±0.54 c	156.14±0.67 c
Chit-40 kDa	S	– As	142.28±1.90 abc	138.29±0.83 ab	137.65±0.54 a	138.4±0.30 ab	141.52±0.46 abc	144.78±0.52 c	143.63±1.12 bc	141.27±2.30 abc
	S	+ As	188.85±0.63 d	187.36±0.39 d	187.23±0.38 de	185.9±0.38 d	186.13±0.10 d	186.19±0.08 d	184.39±0.40 de	184.38±0.92 d
Chit-35 kDa	S	– As	116.20±1.14 a	117.65±0.59 a	116.11±1.03 a	116.1±0.43 a	116.82±0.99 a	118.81±1.56 ab	123.36±0.68 b	116.14±1.09 a
	S	+ As	149.52±0.22 c	148.63±0.58 c	148.62±0.2 c	147.83±0.34 c	148.79±0.50 c	148.9±0.50 c	147.13±0.92 c	146.46±0.93 c
AcChit-A	R	– As	140. 17±1. 13 ab	149. 98±1. 99 cd	147. 36±1. 73 ad	154. 44±0. 55 bed	156. 03±0. 42 bed	158. 27±1. 89 bc	161. 02±2. 46 b	155. 83±1. 70 bcd
	R	+ As	164. 63±5. 85 cde	173. 36±1. 22 cde	174. 36±2. 71 cde	174. 37±1. 75 cd	177. 86±2. 22 bcd	179. 72±3. 22 cde	180. 14±30 bcd	176. 32±7. 62 cde
AcChit-B	R	– As	138. 88±2. 46	141. 4±1. 01	135. 45±1. 15	138. 39±0. 22	137. 04±0. 32	137. 54±0. 84	138. 25±0. 54	137. 91±0. 51
	R	+ As	155. 76±6. 86	168. 57±2. 60	156. 62±2. 86	158. 99±0. 96	155. 67±2. 56	158. 99±3. 58	163. 34±3. 49	159. 64±5. 88
AcChit-C	R	– As	132. 46±1. 27 ab	132. 98±1. 73 b	127. 12±2. 59 ab	131. 74±0. 75 ab	131. 24±0. 63 ab	132. 48±1. 60 b	133±1. 27 b	131. 46±1. 46 b
	R	+ As	118. 30±0. 09 dc	122. 52±1. 52 d	112. 12±0. 85 ab	111. 04±2. 90 ab	109. 14±3. 41 ab	115. 78±1. 14 abc	119. 12±0. 71 cd	118. 38±0. 32 bcd
AcChit-D	R	– As	135. 65±1. 65 a	135. 08±0. 27 a	129. 59±1. 73 a	134. 32±0. 66 a	131. 07±1. 22 a	129. 83±3. 42 a	130. 2±3. 91 a	130. 24±2. 10 a
	R	+ As	149. 66±3. 56 a	152. 4±1. 76 ab	144. 8±0. 98 a	148. 72±1. 08 ab	143. 16±1. 13 a	145. 84±0. 54 a	148. 97±1. 38 a	147. 57±2. 19 a
AcChit-A	S	– As	170. 35±4. 26 ab	172. 78±4. 08 ab	174. 26±1. 91 ab	173. 45±0. 87 ab	171. 22±1. 73 ab	172. 1±0. 95 ab	168. 35±1. 72 a	169. 85±2. 59 ab
	S	+ As	185. 13±0. 44 bc	181. 95±0. 94 abc	184. 94±1. 10 bc	185. 53±0. 43 bc	192. 36±1. 11 c	191. 27±1. 57 c	189. 67±1. 33 c	190. 08±1. 25 c

Table S2. Continued.

AcChit-B	S	– As	168. 80±6. 27 abc	169. 97±3. 41 ab	168. 53±1. 22 a	168. 07±0. 44 a	165. 92±0. 87 a	169. 56±0. 82 ab	166. 96±1. 25 a	166. 87±1. 14 a
	S	+ As	180. 08±0. 77 bcde	176. 32±1. 29 abcd	180. 79±1. 46 cde	180. 03±0. 24 bcde	189. 05±1. 35 e	188. 5±1. 24 e	185. 62±0. 76 de	188. 02±0. 46 de
AcChit-C	S	– As	161. 53±4. 74 a	161. 06±1. 69 a	162. 52±0. 87 ab	167. 42±0. 46 abcde	162. 26±0. 58 ab	165. 47±0. 31 abcd	163. 11±0. 62 ab	163. 69±2. 32 abc
	S	+ As	174. 05±0. 14 bcdef	173. 22±0. 45 abcdef	175. 55±1. 13 cdef	175. 66±0. 13 cdef	181. 39±0. 52 f	179. 71±0. 30 ef	177. 84±0. 10 def	180. 22±0. 64 f
AcChit-D	S	– As	156. 50±4. 81 bcde	157. 41±3. 26 bcde	158. 96±1. 96 bcde	162. 34±0. 60 bcde	157. 8±2. 04 cde	160. 11±1. 09 e	158. 61±1. 49 de	159. 23±1. 95 e
	S	+ As	151.03 abcde	150.06 abc	151.69 abc	152.11 abc	153.81 abcde	153.75 abcd	153.86 ab	155.56 a
BasicChit-a	R	– As	117. 46±2. 98 abc	124. 12±1. 83 abc	121. 61±1. 42 abc	127. 43±1.01 abc	126. 89±0. 37 abc	126. 21±2. 38 bc	128. 15±2. 25 c	127. 23±0. 94 abc
	R	+ As	113. 10±1. 51 a	116. 47±0. 57 abc	114. 44±1. 50 ab	119. 19±0. 93 abc	117. 83±0. 75 abc	116. 56±1. 20 abcd	123. 32±2. 07 abc	125. 38±1. 88 abc
BasicChit-b	R	– As	116. 07±2. 37 abc	126. 08±2. 12 cde	120. 24±2. 45 bcd	129. 29±1. 33 de	127. 49±0. 93 de	130. 44±1. 62 de	132. 81±1. 70 e	131. 34±0. 70 ef
	R	+ As	110. 42±1. 84 ab	117. 97±1. 52 a	110. 97±2. 50 ab	122. 62±1. 03 ab	117. 49±2. 19 abc	118. 22±2. 15 abcd	126. 97±0. 44 abc	123. 11±1. 41 abc
BasicChit-a	S	– As	129. 57±2. 85	129. 3±2. 34	126. 91±1. 31	125. 55±0. 68	126. 64±1. 15	133. 94±2. 24	131. 07±1. 89	131. 82±2. 55
	S	+ As	128. 55±3. 72	126. 77±2. 14	131. 82±0. 37	134. 8±0. 79	135. 75±1. 41	135. 97±1. 02	130. 34±2. 51	126. 72±2. 94
BasicChit-b	S	– As	104. 43±0. 14	102. 63±0. 35	100. 43±0. 16	101. 15±0. 13	106. 63±1. 05	107. 08±1. 13	102. 93±1. 35	98. 04±2. 15
	S	+ As	103. 43±0. 15	101. 63±0. 65	102. 53±0. 10	104. 05±0. 03	106. 93±1. 25	106. 08±1. 02	101. 93±1. 25	99. 04±2. 40
BasicChit-c	S	– As	104. 17±0. 78	101. 23±1. 95	101. 57±0. 39	105. 31±0. 69	110. 11±4. 02	111. 54±3. 01	104. 64±1. 33	106. 54±0. 44
	S	+ As	105. 17±0. 78	100. 46±1. 95	102. 96±0. 39	106. 46±0. 69	111. 1±4. 02	110. 64±3. 01	106. 74±1. 33	107. 21±0. 44
BasicChit-d	S	– As	111. 52±2. 88 ab	110. 27±2. 46 abc	112. 32±1. 49 ab	113. 12±0. 66 e	114. 62±0. 55 bc	118. 21±1. 57 bc	117. 51±2. 46 e	118. 36±2. 82 e
	S	+ As	105. 75±1. 01 b	102. 32±0. 05 bc	103. 31±0. 33 bc	103. 53±0. 05 bcde	105. 76±0. 52 cd	104. 09±1. 09 cd	99. 88±2. 40 cd	96. 18±1. 37 cde

* Activity of total glucanases was expressed as mmol of released glucose per mg of soluble protein sample per hour. Activity of chitinases was expressed as picomoles of methylumbelliferone generated per microgram of soluble protein per hour. The activities of individual isoforms correspond to background-corrected integrated density of the enzyme bands in polyacylamide gels.