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Arsenic exposure modifies *Fusarium* wilt tolerance in grass pea (*Lathyrus sativus* L.) genotypes through modulation of antioxidant defense response

Dibyendu Talukdar

Correspondence: dibyendutalukdar9@gmail.com

Plant Cell and Stress Biology Laboratory, Department of Botany, R.P.M. College, University of Calcutta, Uttarpara, Hooghly 712258, West Bengal, India.

Abstract

Background: Arsenic (As) is a toxic metalloid for plant growth while wilt caused by *Fusarium* is a major yield-limiting factor in grain legumes. Despite immense threat of escalation of major biotic stresses in crops due to contaminated soil, the intrinsic cellular responses to *Fusarium* wilt under As exposure is not known. Furthermore, the role of primary antioxidant defense other than phenylpropanoid pathway in response to wilt is poorly understood in crop plants.

Methods: Effect of As on wilt tolerance was studied on four genotypes of grass pea (*Lathyrus sativus* L.) (30 days after commencement of treatment) in a controlled pot experiment with randomized block design in four replicates per four treatment: control (no *Fusarium*-inoculums, no As in soil), inoculums-treated soil and As + inoculums-treated soil. Data were statistically analyzed at a significant level of $P < 0.05$.

Results: In inoculums-treated soil, glutathione (GSH)-deficient *gshL-1* mutant wilted but wilting symptom was conspicuously absent in variety BioL-212, ascorbate (AsA)-deficient mutant (*asfL-1*), and GSH-overproducing mutant. In presence of As, wilting symptoms appeared in BioL-212 and their severity increased in the *gshL-1* mutant. Normal activity of phenylalanine ammonia-lyase (PAL) in susceptible *gshL-1* mutant and remarkable tolerance of *asfL-1* mutant to wilt despite low PAL activity, during As exposure, indicated involvement of antioxidant defense other than PAL in governing wilt sensitivity. Enhanced peroxidase level along with high GSH-redox and low ascorbate peroxidase activity tightly regulated H_2O_2 concentration and metabolism in fine tune, effectively preventing oxidative damage of membrane and concomitant escalation of wilting symptom. Low H_2O_2 level and deficient peroxidase activity, presumably, weakened *gshL-1* defense against fungal invasion. By contrast, under As + inoculums-treated soils, H_2O_2 level reached toxic concentration in the mutant and also in variety BioL-212 resulting in onset of oxidative stress and appearance of wilting symptom in the severest form.

Conclusions: As has profound effect on PAL activity and induced/increased wilt symptom through modulation of H_2O_2 metabolism within AsA-GSH cycle and outside it. H_2O_2 level was found critical in determining tolerance or sensitivity of genotypes to *Fusarium* wilt, and the GSH-redox state holds the key to manage oxidative damage during pathogenic infection.

Keywords: *Fusarium* wilt, arsenic, phenylalanine ammonia-lyase, AsA-GSH cycle, redox state, peroxidase, H_2O_2 , *Lathyrus sativus* L

Introduction

Phenylalanine ammonia-lyase (PAL) catalyzes the first step in the phenylpropanoid pathway, by deamination of L-phenylalanine to trans-cinnamate and ammonia [1]. In addition to its important roles in plant development, PAL is also a key enzyme in plant stress responses. Its expression and activity is stimulated by diverse types of biotic and abiotic stress factors such as pathogen attack, tissue wounding, extreme temperatures, UV irradiation, nutrient deficiency, plant signaling molecules, including jasmonic acid, salicylic acid and abscisic acid [2,3], salinity [4] and heavy metal toxicity [5]. The production of reactive oxygen species (ROS) is one of the earliest cellular defense responses following successful pathogen recognition [6,7]. ROS production is a usual process in an aerobic cell but it may be unbalanced in favor of oxidative state during onset of stress induced by biotic as well as abiotic factors [8]. Ascorbate (AsA)-glutathione (GSH) cycle plays

a fundamental role in cellular detoxification of ROS, in which ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) enzymes scavenge hydrogen peroxide (H_2O_2), and regenerate AsA and GSH, respectively [8-10]. Nonspecific peroxidases (POX) also function efficiently in H_2O_2 metabolism, utilizing diverse substrates during plant growth [10]. Although ROS production, such as hydrogen peroxide (H_2O_2) and superoxide radicals, has been considered as a prominent defense against phyto pathogen attack [6,7], it must be maintained in a level compatible with plant growth and development [6,9,10]. The redox state of both AsA and GSH is far more influential than their amount during stress response [8].

Arsenic (As) is a ubiquitous toxic metalloid without biological functions in higher plants. Being an analogue of phosphate, arsenate is able to compete and is readily taken up by plants through high-affinity phosphate transporters of root membranes [11].

In recent times, the impact of irrigation with high As contaminated ground water on soil and crops has drawn huge attention due to transfer of As to the food chain via the groundwater-soil-plant system [12]. Grass pea (*Lathyrus sativus* L.) is a cool-season legume crop, cultivated in a very low-input condition for both food and fodder in the Indian sub-continent, sub-Saharan Africa, Mediterranean regions, South America, parts of Europe and Australia [13] and this crop undergoes diverse types of biotic and abiotic stresses [14]. Growing evidences indicate that As can be significantly accumulated in grain and forage legumes [15,16] and it induces oxidative stress [17-19]. Recent reports indicated that As-exposure greatly facilitated invasive potentials of *Leucaena* sp and increased weed-induced phytotoxicity in grass pea and lentil [20,21]. In grass pea, As-exposure significantly reduced seed germination, inhibited photosynthetic activities and plant growth but significantly enhanced the level of seed neurotoxin, β -ODAP (β -N-Oxalyl-L α , β -diamino propionic acid) [22,23]. In recent years, promising mutant lines and genotypes exhibiting novel cytogenetic features, desirable agronomic traits and particular mechanism of antioxidant defense have been isolated and characterized in grass pea [24-32]. Mutational strategy provides a powerful tool to analyze the responses of various *Arabidopsis* mutants like *cad2*, *rax1-1* and *pad2* and their double and triple mutants to various bacterial, fungal and oomycete pathogens as well as to insects [33-35]. Roles of antioxidant defense components like flavonoids, ascorbate-glutathione cycle enzymes, and catalase have been elucidated in legume mutants [36,37], and have been mapped along with morphological and isozyme markers [38,39].

Wilt caused by *Fusarium* spp is a major biotic stress factor for low crop productivity and yield instability in various food legumes including grass pea [40]. *F. oxysporum* is a soil borne, root pathogen colonizing the vessels and blocking them completely to effect wilting [40]. It affects the susceptible cultivars within 20 days after sowing and infected seedlings exhibit drooping of leaves, xylem and stem discoloration and root rotting followed by complete collapse. Major grass pea growing areas in several countries including Indian sub-continent are now As-contaminated where yield losses by *Fusarium* wilt are also prevalent [41]. However, it is unclear whether As-exposure can induce/escalate *Fusarium* wilt in grass pea cultivated in contaminated soil, and if so, whether PAL activity is exclusively involved in intrinsic modulation of plant response to wilting. Mutation analysis revealed major insight during antioxidant defense responses to pathogenecity in *Arabidopsis* [8]. Evidence that glutathione is involved in defense against biotic stress came from the studies of *Arabidopsis* mutants altered in GSH synthesis or metabolism [8]. Despite immense possibility of induction/escalation of biotic stress conditions under As-exposure, no similar information is available regarding agronomically important plant species. Furthermore, virtually nothing is known about involvement of primary antioxidant defense other than PAL during plant-

pathogen interaction in contaminated soil. The present study was therefore undertaken to ascertain the effect of As on wilting phenomenon of grass pea genotypes and to explore the roles of PAL and AsA-GSH-cycle enzymes as well as AsA and GSH-redox in modulation of plant response to wilting under As-exposure. The present investigation should provide the first evidence that PAL activity is not exclusively involved in modulation of plant response to *Fusarium* wilt in legume crop rather PAL activity was modulated through a well integrated response of AsA-GSH cycle enzymes and peroxidase in H_2O_2 -metabolism as evidenced by the response of four genotypes to *Fusarium* wilt.

Materials and methods

Collection and inoculums preparation

Culture of *Fusarium oxysporum* f. sp. *lentis* causing wilt disease in grass pea and lentil was obtained from a local agricultural experimental station, Kalyani, India. The pathogen inoculum was prepared by culturing the fungus on potato dextrose agar medium for 7 days in Petri dishes at $25 \pm 1^\circ\text{C}$. The micro conidial suspension was prepared by pouring 20 ml of sterile distilled water in each Petri dishes. The concentration of micro-conidia was adjusted to 1000 conidia ml^{-1} .

Plant materials and growth conditions

Dry and healthy seeds of four grass pea (*Lathyrus sativus* L.) genotypes were used and namely one ascorbate (AsA)-deficient semi-dwarf mutant (*asfL-1*) containing only 42% of leaf and 20% of root AsA content of variety BioR-231 [42], one glutathione (GSH)-deficient mutant (*gshL-1*) with 15% of total glutathione in leaves and 20% of that in roots of variety BioL-212 [43] and one GSH-overproducing mutant exhibiting >2.5-fold increase in foliar GSH-content and enhanced antioxidant capabilities compared to its mother variety BioR-231 [44]. Seeds were surface-sterilized with 0.01% aqueous HgCl_2 for 1-2 min, thoroughly washed with double distilled water and then are put to germinate. The grass pea variety BioL-212 has been used as mother variety in the present experiment. Five germinated seeds per genotypes were sown in 30 cm pots, filled with air dried soil (5.5 kg, top soil of 0–20 cm of Alfisols sampled from Gangetic West Bengal, India, 15 m above sea level, $88.35^\circ\text{E}/22.67^\circ\text{N}$, clay_alluvial brown soil, pH 6.3, soil : water ratio of 1 : 2.5, organic matter 5.17 g/kg soil, Kjeldahl N 750 mg/kg soil, P_{Olsen} 14.18 mg/kg soil, available K 77.67 mg/kg, available S 13.80 mg/kg) [18], and after 10 days of growth they were finally thinned to two seedlings per pot. The plants (10 d-old) were, then, subjected to the following four treatments: (a) control (all four genotypes in non-infested soil and no arsenate), (b) inoculums-treated (pot filled with sterilized *F. oxysporum* infested soil at the rate of 100 ml homogenized culture per pot, 5 day before planting), (c) 50 μM sodium arsenate (As, MW 312.01 g/mol; technical grade, purity 98.5%, Sigma-Aldrich, Bangalore, India) added in already infested soil, and (d) As + plant. Pilot experiment was carried out to

differentiate sensitive genotypes from tolerant ones under combined exposure of As and inoculum, and 50 μM of As was found most effective to generate response which can be used as determining criteria between sensitive and tolerant genotypes, and thus was selected for the present study. The pots were arranged in randomized block design in controlled growth chamber under a 14-h photoperiod, 28/18 ($\pm 2^\circ\text{C}$), relative humidity of $70 \pm 2\%$ and a photon flux density of $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ in four replicates per treatment. Control and treated plants were allowed to grow for another 30 d and data were recorded as days after commencement of treatment (DAC). Pots were refreshed every two days to prevent depletion of biotic (inoculums) and abiotic (As) inducers in the course of the plant's exposure to them. Normal agricultural practices were performed. Plants were harvested and shoot tissues were used for further assays.

Enzyme extraction and activity assay

Fresh shoot tissue (250 mg) was homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 2% (w/v) polyvinyl pyrrolidone (PVP) using chilled mortar and pestle kept in ice bath. The homogenate was centrifuged at 15,000 g at 4°C for 30 min. Clear supernatant was used for enzyme assays. For measuring APX (ascorbate peroxidase) activity, the tissue was separately ground in homogenizing medium containing 2.0 mM ascorbate in addition to the other ingredients. All assays were performed at $0-4^\circ\text{C}$. Soluble protein content was determined according to Bradford [45] using Bovine Serum Albumin as a standard.

APX (EC: 1.11.1.11) activity was assayed following Nakano and Asada method [46]. Three milliliters of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM AsA, 0.1 mM H_2O_2 and 0.1 ml of enzyme extract. The hydrogen peroxide-dependent oxidation of AsA was followed by a decrease in the absorbance at 290 nm ($\epsilon = 2.8 \text{ mM}^{-1}\text{cm}^{-1}$). APX activity was expressed as $\mu\text{mol AsA oxidized min}^{-1} \text{ mg}^{-1} \text{ protein}$.

DHAR (EC 1.8.5.1) activity was measured following the protocol of Nakano and Asada [46]. The complete reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.2 mM DHA and 0.1 mM EDTA in a final volume of 1 ml. Reaction was started by adding suitable aliquots of enzyme extract and the increase in absorbance was recorded at 30 s intervals for 3 min at 265 nm. Enzyme activity was expressed as $\mu\text{mol AsA formed min}^{-1} \text{ mg}^{-1} \text{ protein}$.

Glutathione reductase (GR, EC: 1.6.4.2) activity was determined by monitoring the glutathione dependant oxidation of NADPH, as described by Carlberg and Mannervik [47]. In a cuvette, 0.75 ml 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA, 75 μl NADPH (2 mM), and 75 μl oxidized glutathione (20 mM) were mixed. Reaction was started by adding 0.1 ml enzyme extract and the decrease in absorbance at 340 nm was monitored for 2 min. GR specific activity was calculated as nmol NADPH oxidized $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$ using

an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

POX (EC 1.11.1.7) was measured by monitoring oxidation of 4 mM guaiacol at 470 nm ($\epsilon = 22.6 \text{ mM}^{-1} \text{ cm}^{-1}$) in 50 mM potassium-phosphate buffer (pH 6.5), after addition of 1 mM H_2O_2 [42]. Enzyme activity was expressed as $\mu\text{mol mg}^{-1}(\text{protein}) \text{ min}^{-1}$.

Phenylalanine ammonia-lyase (PAL, EC: 4.3.1.5) activity was determined following the direct spectrophotometric method adopted by Cavalcanti et al., [48]. Two hundred microlitres of the crude enzyme extract previously dialyzed overnight with 100 mM Tris-HCl buffer, pH 8.8, were mixed to a solution containing 200 μl 40 mM phenylalanine, 20 μl 50 mM β -mercaptoethanol and 480 μl 100 mM Tris-HCl buffer, pH 8.8. After incubation at 30°C for 1 h, the reaction stopped by adding 100 μl 6 N HCl. Absorbance at 290 nm was measured and the amount of trans-cinnamic acid formed was evaluated by comparison with a standard curve (0.1-2 mg trans-cinnamic acid/ml) and expressed as μg of trans-cinnamic acid $\text{h}^{-1} \text{ mg}^{-1} \text{ protein}$.

Determination of AsA and GSH content and their redox states

Reduced and oxidized form of ascorbate and glutathione were measured according to methods of Law et al., [49] and Griffith [50], respectively, and their redox state was calculated as AsA/(AsA+DHA) and GSH/(GSH+GSSG), respectively.

Determination of H_2O_2 content and lipid peroxidation level

H_2O_2 was estimated following the method of Wang et al., [51]. Fresh tissue (0.1 g) was powdered and blended with 3 ml acetone for 30 min at 4°C and was filtered through eight layers of gauze cloth. After addition of 0.15 g active carbon, the sample was centrifuged twice at 3,000 g for 20 min at 4°C , then 0.2 ml 20% TiCl_4 in HCl and 0.2 ml ammonia was added to 1 ml of the supernatant. After incubation for 10 min, the compound was centrifuged at 3,000 g for 10 min, the supernatant was discarded and the pellet was dissolved in 3 ml of 1 M H_2SO_4 and absorbance was measured by UV/Vis spectrophotometer (PerkinElmer Lambda 25, Mumbai, India) at 410 nm. H_2O_2 content was measured from the absorbance at 410 nm using a standard curve. Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents according to method of Hodges et al., [52]. About 0.5 g of fresh tissue was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3,000 g for 12 min at 4°C to pellet debris. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was mixed either with 20% trichloroacetic acid (TCA) or with a mixture of 20% TCA, 0.65% thiobarbituric acid and 0.01% butylated hydroxy toluene (BHT). Both mixtures were allowed to react in a water bath at 95°C for 25 min, cooled to room temperature and centrifuged. Absorbance was measured at 450, 532 and 600 nm against a blank. Level of lipid peroxides was calculated following Hodges et al., [52]

Figure 1. Healthy flowering twig of un-treated grass pea *gshL-1* mutant (A) and appearance of severe wilting symptoms exhibiting tuft of rolled crinkled terminal leaflets (bold arrow) and rudimentary leaflets (thin arrow) in the mutant grown in inoculum-treated pot soil (B), and appearance of wilting phenotype in grass pea variety BioL-212 (C) and characteristic rolling of all leaflets in *gshL-1* mutant (D) under As (sodium arsenate) + inoculum-treated pot soil.

and expressed as nmol MDA g⁻¹ fresh weight.

Statistical analysis

The results presented are the mean values $\pm$ standard errors of at least four replicates. Multiple comparisons of means were performed by ANOVA (SPSS Inc. v. 10), and the means were separated by Duncan's multiple range test considering significant differences at $P < 0.05$.

Results

Effect of *Fusarium* inoculums and inoculums + As treatment on plant morphology

All the four genotypes of grass pea were quite normal under un-treated condition. In inoculum-treated set, wilting symptom was not observed in mother variety BioL-212 and in *asfL1*

and *GSH-overproducing* mutants. However, symptoms of *Fusarium* wilt such as dropping of leaflets, small rudimentary leaflets, extremely wrinkled tuft of terminal leaflets with dense green color and stunted internodes were evident on shoots of 30-d-old *GSH-deficient gshL-1* mutant exposed to inoculums (**Figure 1A,B**).

In As + inoculum-treated soil, mother variety developed severe wilting which was initially manifested as dropping of leaflets on 20 DAC and consequently, as collapse of upper shoot portions with wrinkled leaflets on 30 DAC (**Figure 1C**). In *gshL-1* mutant, the appearance of symptoms were anticipated by 10 days and exhibited as characteristic leaf rolling (**Figure 1D**), wilting of shoot, withering of leaflets and stipules, and bud drop out on 20 DAC. Wilting symptom was totally absent in *asfL-1* mutant and *GSH-overproducing* mutant. No wilting symptom was observed in any of the four genotypes subjected to As + plant treatment, and thus, the effects of rest three treatment protocols on wilting will be discussed here.

Effect of *Fusarium* inoculums and inoculums + As treatment on PAL activity

In inoculum-infested soil without any As, PAL activity was increased in comparison with respective control about 2-fold in BioL-212, nearly 2.5-fold in *asfL-1* and about 3-fold in shoots of *GSH-overproducing* lines at harvest (**Figure 2A**). Enzyme activity in all genotypes started to rise at 10DAC, reached its peak at 20 DAC, and then became stationary (data not shown). Compared to control, marginal increase in PAL activity was observed in inoculum-treated *gshL-1* shoots (**Figure 2A**). Under exposure to As + inoculum-treated soil, PAL activity was decreased significantly in BioL-212, *asfL-1* mutant and *gshL-1* shoots in comparison with respective control, while its level was maintained high in *GSH-overproducing* mutant (**Figure 2A**).

AsA-GSH cycle enzyme and POX activity in inoculum-treated and As + inoculum-treated materials

Excluding *gshL-1* mutant, a substantial decrease of APX activity was observed in BioL-212 and *GSH-overproducing* mutant exposed to inoculums. Compared to control, APX activity was increased by about 5-fold in *gshL-1* mutant (**Figure 2B**). The treatment with As + inoculums increased APX activity over control by about 2-fold in BioL-212 but a decrease of 4-4.3-fold in *gshL-1* and *GSH-overproducing* mutant was detected (**Figure 2B**). Compared to control, APX activity was non-significantly ($P > 0.05$) changed in *asfL-1* shoot in both treatments.

Exposure to inoculums led to significant increase in GR activity in BioL-212 and *asfL-1*, while it was similar to control in *GSH-overproducing* mutant and decreased by 2.7-fold in *gshL-1* mutant (**Figure 2C**). The treatment with As + inoculums resulted in considerable increase in GR activity in *asfL-1* and *GSH-overproducing* mutant (> 3.0 -fold) but the activity decreased by 3.5-fold in BioL-212 and nearly 3-fold in *gshL-1* mutant

Figure 2. Activities of phenylalanine ammonia-lyase (PAL) (A), ascorbate peroxidase (APX) (B), glutathione reductase (GR) (C), and dehydroascorbate reductase (DHAR) (D) in grass pea variety BioL-212, and three mutant lines, *asfL-1*, *gshL-1* and *GSH-op* (*GSH-overproducing*) grown in un-treated control, inoculums-treated and As (sodium arsenate) + inoculums-treated pot soil. Vertical bar represents means ($n = 4$) $\pm$ SE. Means followed by the same letter were not significantly different at $P < 0.05$ by ANOVA followed by Duncan's Multiple Range Test.

(**Figure 2C**). DHAR activity was constitutively low in *asfL-1* mutant under un-treated and treated conditions. It was comparable among other genotypes under inoculums-treated condition. The treatment with As + inoculums enhanced DHAR activity over the control nearly 2-fold in both BioL-212 and *GSH-overproducing* mutant but decreased the activity by 1.8-fold in *gshL-1* mutant (**Figure 2D**).

In comparison to control, activity of guaiacol-peroxidase was 4-fold lower in *gshL-1* mutant but it was quite similar in other genotypes exposed to inoculums-treated soil. Treatment with As + inoculums markedly increased (about 3-fold) enzyme activity in *asfL-1* shoot. POX activity was unaffected in *GSH-overproducing* mutant but it was decreased significantly in BioL-212 and *gshL-1* mutant (**Figure 3A**).

Figure 3. Activities of guaiacol-peroxidase (POX) (A), and reduced ascorbate (AsA) (B), AsA-redox (C), reduced glutathione (GSH) content (D), oxidized glutathione (GSSG) (E) and GSH-redox (F) in seedlings of grass pea variety BioL-212, and three mutant lines, *asfL-1*, *gshL-1* and *GSH-op* (*GSH-overproducing*) grown in un-treated control, inoculums-treated and As (sodium arsenate) + inoculums-treated pot soil. Vertical bar represents means (n = 4) ± SE. Means followed by the same letter were not significantly different at P < 0.05 by ANOVA followed by Duncan's Multiple Range Test.

Figure 4. H₂O₂ (A) and MDA (B) level in seedlings of grass pea variety BioL-212, and three mutant lines, *asfL-1*, *gshL-1* and *GSH-op* (*GSH-overproducing*) grown in un-treated control, inoculums-treated and As (sodium arsenate) + inoculums-treated pot soil. Vertical bar represents means (n = 4) ± SE. Means followed by the same letter were not significantly different at P < 0.05 by ANOVA followed by Duncan's Multiple Range Test.

Effect of *Fusarium* inoculums and inoculums + As treatment on AsA and GSH redox

The AsA content and AsA-redox state did not change significantly in all the four genotypes upon exposure to

inoculums but they were fallen by about 2-fold in *asfL-1* and about 3-fold in *gshL-1* mutants exposed to As + inoculums treatment (**Figure 3B,C**). The GSH-redox was unchanged in BioL-212 when infected with inoculums alone but it was reduced in As + inoculums treated soil. GSH content was significantly higher in *asfL-1* mutant than other three genotypes and it was affected non-significantly in both treatments (**Figure 3D**). GSH content was reduced significantly in *gshL-1* mutant, exposed to inoculums and a further reduction was induced by As + inoculums treatment (**Figure 3D**). By contrast, GSSG amount was markedly enhanced in inoculums-treated BioL-212 and As + inoculums-treated *gshL-1* mutant (**Figure 3E**). GSH-redox was quite high in *asfL-1* and *GSH-overproducing mutant*, hovering around 0.9 in both treatment conditions (**Figure 3F**). Although deficient in total content, GSH-redox was similar to control in *gshL-1* mutant in un-treated condition, but it was considerably decreased (3-fold) in seedlings grown in infested soil and even more when As was added in soil (**Figure 3F**). The decrease in redox state of both AsA and GSH was mainly carried out by a lowering of their reduced form and/or concomitant increase in their oxidized form (**Figure 3F**).

Hydrogen peroxide (H₂O₂) and lipid peroxidation (malondialdehyde or MDA) contents

Shoot H₂O₂ of BioL-212, *asfL-1* and *GSH-overproducing mutant* was increased by about 2.2-fold, 2-fold and 2.5-fold, respectively, in presence of inoculums alone (**Figure 4A**). Compared to control, shoot H₂O₂ content was significantly lower in *gshL-1* mutant grown in inoculums-treated pot soil. Upon exposure to As + inoculums, H₂O₂ level was further increased in BioL-212 but was remained unchanged in *asfL-1* and *GSH-overproducing mutant*. By contrast, there was nearly 6-fold rise in tissue H₂O₂ level in *gshL-1* mutant exposed to As + inoculums-treated soil (**Figure 4A**).

Measureable MDA content was similar in BioL-212, *asfL-1* and *GSH-overproducing mutant* but it was significantly higher than control in *gshL-1* mutant grown in inoculums-treated soil (**Figure 4B**). However, a significant rise in MDA content was observed in both BioL-212 and *gshL-1* mutant with more increase in case of the mutant under As + inoculums-treated soils (**Figure 4B**). Negligible changes in MDA level were noticed in *asfL-1* and *GSH-overproducing mutants* when compared with control (**Figure 4B**).

Discussion

Fusarium wilt is a severe biotic stress factor for yield losses in grain legumes, fruits, vegetables and other economically important crop plants [53,54]. In the present study, four grass pea genotypes with contrasting antioxidant defense backgrounds were considered to study the modulation of phenylpropanoid pathway and AsA-GSH cycle, the role of H₂O₂-scavenging enzymes and of two non-enzymatic antioxidants, the AsA and GSH during exposure of *Fusarium* inoculums and As + inoculums. In the first pot experiment, in controlled

and uniform growth condition, results revealed that infested soil triggered major wilting symptoms only in *gshL-1* mutant while the other three genotypes did not show significant changes in morphology. Conspicuous absence of wilting in all four genotypes exposed to As alone but the occurrence of wilting in *gshL-1* mutant exposed to inoculums only and also increasing its severity under As + inoculums + plant treatment suggested that wilting symptom was not due to the effect of metalloid but its presence in soil increased the severity of wilting caused by *Fusarium*. This was further substantiated by the fact that BioL-212 showed no wilting in As + plant and inoculums + plant treatments but the plant wilted when As was added along with inoculums in soil. The results suggested apparent tolerance of mother variety BioL-212 and the two mutants, *asfL-1* and *GSH-overproducing* to imposed doses of *Fusarium* inoculums but indicated a strong susceptibility of *gshL-1* mutant to the disease.

In order to ascertain the differential response of four genotypes to *Fusarium* inoculum, activities of five prominent antioxidant enzymes in plants were tested. Significant increase of PAL activity in BioL-212, *asfL-1* and *GSH-overproducing* mutant strongly suggested pathogen-induced rise of the enzyme activity in conferring protection against wilt. PAL activity was enhanced in wheat plants treated with BTH, a novel systemic acquired resistance inducer in response to powdery mildew infection [55], in ASM-primed resistance in Japanese pear [56] and cucumber [57]. Enhanced PAL activity was also reported in chickpea seedlings treated with *Rhizobium* Pch43 followed by *F. oxysporum* f. sp. *ciceri* race 0 and chemical as well as biological inducers [58], in SA-primed as well as *Bacillus*-treated tomato plants in response to *Fusarium oxysporum* [59] and in other crop plants subjected to elicitors and inducers [57-59]. In the present experiment, no external priming or stimuli was used to elicit PAL activity against *Fusarium* inoculums and no constitutive over-expression of PAL was observed in all considered genotypes under un-treated control. This finding confirmed responsiveness of PAL-mediated primary defense system against *Fusarium* wilt and also indicated that strain specific to lentil was also able to induce pathogenic response in grass pea.

The susceptibility of *gshL-1* mutant to *Fusarium* wilts despite normal PAL activity was surprising and unique in crop plants, and is not in accordance with the common opinion that in response to invasion of pathogen, PAL activity is enhanced. In order to clarify this apparent conflicting situation and to reveal the involvement of other primary defense mechanism during *Fusarium* infection, role of defense components of AsA-GSH-cycle and H_2O_2 -scavenging was investigated in four genotypes of grass pea. These four genotypes, as reported in plant material section, were selected for their distinct differences in antioxidant defense mechanism. In particular mother variety BioL-212 did not show significant differences in redox state of both AsA and GSH and enzyme activities, the *asfL-1* mutant was highly deficient in AsA-redox and *gshL-1*

was deficient in GSH level, while high redox value of GSH was observed in *GSH-overproducing* mutant in un-stressed condition. More importantly, these three mutant traits are constitutively expressed and inherited as novel stable traits in advanced mutant progenies [42-44]. The primary and significant defense responses of these three mutants are GSH-mediated modulation of H_2O_2 -metabolism within AsA-GSH cycle and outside it [42-44]. Novelty of different mutants has also been used in model plant *Arabidopsis thaliana* to dissect antioxidant defense response during plant-pathogen interaction [60]. The results obtained in the present investigation are interesting. In inoculums-treated soil, significant high level of APX and declining activity of POX in *gshL-1* mutant suggested a possible involvement of H_2O_2 in regulating its disease resistance. Role of H_2O_2 as a diffusible ROS in protection against diverse types of pathogen attack and hypersensitive response has been elucidated in different crop plants [7,61,62]. Association of H_2O_2 with cell wall lignifications, callose synthesis, cell wall cross-linking and in formation of other physical defensive barriers was found during fungal infections [62]. The resistant iso-line Williams-8818-1 of banana exhibited a typical response of first "oxidative burst" during *Fusarium* infection, in which H_2O_2 accumulation was greatly induced [7]. The opposite response of two peroxidases in the *gshL-1* mutant considered indicated that H_2O_2 is scavenged vigorously by enhanced activity of APX while POX activity was not enough to take part in mechanical strengthening of shoot tissue required to prevent wilt during *Fusarium* invasion. Role of GR in this process is also worth mentioning. Low GR activity, affecting GSH recycling resulted in abnormal low level of GSH-redox in *gshL-1* shoot which inhibited GSH-mediated antioxidant defense. Significant high MDA content suggested onset of oxidative damage of shoot membrane despite normal DHAR activity and AsA-redox in inoculums-treated *gshL-1* mutant and strongly indicated that AsA-GSH cycle enzymes and GSH-redox are more involved rather than AsA during pathogen infection. This observation was further confirmed by the facts that in other three genotypes exhibiting tolerance to wilting APX activity was low but POX and GR activities along with GSH-redox were normal to high. H_2O_2 has the ability to modulate the activity of APX and POX in stressed cell [42], and its dual role as stress-inducer and in ROS-signaling to up-regulate antioxidant defense has been elucidated in different crop plants [9,10,18,19]. In tomato, salicylic acid-mediated inactivation of H_2O_2 -degrading enzymes invoked hypersensitive cell death. Similar situation was reported in jasmonic acid-treated pepper and tomato plants during *Fusarium* infection [61]. Presumably, ROS were proposed to act synergistically in a signal amplification loop with external or internal stimuli to drive the hypersensitive reaction and the establishment of systemic defense [62]. In the present investigation H_2O_2 level was increased in BioL-212, *asfL-1* and *GSH-overproducing* mutant, but MDA level was quite similar in all three cases. Although a positive correlation between rise of H_2O_2 level and

increase in lipid peroxidation has been reported in many cases [10,17, 21,31,37], our results showed an opposite trend. The actual toxicity of ROS in a given plant–pathogen interaction depends on the sensitivity of the pathogen to the concentration of ROS present, but high ROS concentrations are toxic to host plants, too [62]. Obviously, H_2O_2 level in the present genotypes showing resistant to wilts was tightly regulated and it represents a critical control point for modulation of antioxidant enzyme activities in favor of mechanical strength as well as prevention of oxidative damage of membrane. This tight regulation of H_2O_2 accumulation was not observed in *gshL-1* mutant and a declining H_2O_2 content was thus unable to trigger antioxidant defense against pathogen attack in inoculums-treated soil. It is noteworthy that enzymatic metabolism in regulation of H_2O_2 level in stressed plant also depends on redox status of either AsA or GSH or both [8]. Elevated GR activity in BioL-212 and *asfL-1* strongly indicated steady availability of its substrate, the GSSG, produced by DHAR activity when GSH is consumed for detoxification of ROS. GSSG is also generated outside AsA-GSH cycle [42]. Results showed that GSSG level was high in inoculums-treated *gshL-1* mutant, indicating significant consumption of GSH during *Fusarium* infection but the failure of GR activity, due to its constitutively low expression in the mutant led to low GSH-redox which triggered oxidative damage of membrane lipids, as manifested by significantly high MDA content in the treated mutant. By contrast, a normal to high GR activity in three other genotypes showing tolerance to wilting ensured effective regeneration of GSH fundamental in detoxification of lipid peroxides. Present results thus revealed that *Fusarium* infection alone induced oxidative damage as biotic stress factor and GSH-mediated antioxidant defense in addition to PAL activity played vital roles in conferring resistance to wilting in grass pea genotypes.

The sensitivity of four grass pea genotypes to *Fusarium* wilts was significantly changed once 50 μ M As was added in inoculums-treated pot soils. The main objective was to ascertain whether presence of As in soil is able to modify sensitivity and defense mechanism of grass pea genotypes to wilting. Appearance of wilting symptom in BioL-212 and *gshL-1* mutant indicated their susceptibility to *Fusarium* infection under As exposure. The effect was more severe in *gshL-1* seedlings as symptoms appeared 20 DAC compared to BioL-212 where wilting symptoms developed at 30 DAC. By contrast, a normal growth of *asfL-1* and *GSH-overproducing mutant* in the As + inoculums-treated soil suggested tolerance of two mutants to wilts even in toxic soil. The result was not in accordance with earlier reports regarding induction of resistance against *Fusarium* wilting by pre-foliar spray with different chemicals and elicitors in tomato [63], in our results As induced wilt susceptibility in BioL-212 and enhanced the symptoms in *gshL-1* mutant. The phenomenon of wilting in BioL-212 and *gshL-1* mutant and the tolerance in *asfL-1* and *GSH-overproducing mutant* under As exposure were intimately associated with perturbation in intrinsic cellular defense

mechanisms. This was expressed by a significant reduction of PAL activity in BioL-212, *asfL-1* and *gshL-1* mutant and an opposite rise in *GSH-overproducing mutant*. Despite a low PAL level, in *asfL-1* mutant did not develop wilting symptoms under As + inoculums-treated soil. Furthermore, both APX and DHAR activities were also low in the mutant, and AsA-redox was reduced. The remarkable tolerance of this mutant may be related to balanced accumulation of ROS, particularly H_2O_2 level along with high level of GR activity and enhanced POX level. Increased GR activity ensured high GSH-redox through efficient recycling of GSH while over-activity of POX might have utilized excess H_2O_2 in oxidative cross-linking of cell walls. Over-accumulation of H_2O_2 and concomitant increase in membrane damage due to lipid peroxidation are two common symptoms of As-induced oxidative damage and growth inhibition in plants [31,37]. H_2O_2 level was significantly increased in *asfL-1* mutant but it was comparable to that of inoculums-treated condition and MDA content was unaffected. Presumably, in *asfL-1* mutant GSH-redox state rather than PAL activity played key roles in conferring tolerance to As-induced oxidative damage and consequently to fungal infection by maintaining H_2O_2 concentration to a level favorable to plant growth. Similar results are also reported in cadmium-treated lentil seedlings, allelopathic interaction and during phytotoxicity [9,10,17-19,21,64]. Increasing levels of ROS, built up by either enhanced production and decreased scavenging potential, may contribute to the resistance reaction in tomato against *Fusarium* wilt [63]. In *GSH-overproducing mutant*, PAL activity was increased and also POX and GR activities were higher and this may contribute in maintaining normal growth of the mutant through up-regulation of antioxidant defense. On the other hand, increase in severity of wilting symptom in *gshL-1* mutant under As + inoculums-treated soil was associated with further decrease in PAL, GR and POX activity. As exposure also decreased APX and DHAR level as well as AsA-redox, indicating total collapse of all antioxidant defense components in the mutant. Consequently, high H_2O_2 level and MDA content were detected in *gshL-1* shoots determining the highest degree of wilting symptom in comparison with other genotypes tested.

Besides serving as a ROS-regulator, GSH is the exclusive precursor of phytochelatins (PCs), a thiol peptide formed in response to metal stress including As [8], and is used as a reductant by arsenate reductase during conversion of As V to As III in plant tissue [65]. This As III can readily react with thiol-containing enzymes and proteins, and form PC-As III complexes [65]. Although molecular mechanism is still debated, GSH-dependent arsenate reductase activity in the form of glutaredoxin, a ubiquitous oxidoreductase who uses reducing power of GSH to catalyze disulfide reactions in the presence of GR, was manifested in *Arabidopsis*, *Oryza* and *Pteris vittata* exposed to As in different magnitudes [66,67]. Earlier studies revealed that due to low expression of GR activity, GSH as well as PCs level is constitutively low in the present *gshL-1* mutant [43]. Preliminary experiments pointed out significant

lower level of PCs and GSH-S-transferase (GSTs) activity in *gshL-1* mutant during As + inoculums treatment compared to control [Talukdar, unpublished]. Metal chelation through PCs is an important defense strategy in metal homeostasis as PC-deficient *Arabidopsis* mutants and *gshL-1* mutant in grass pea were shown to be hypersensitive to metals like As and cadmium, respectively [43,65]. Obviously, low GSH level in *gshL-1* mutant during As + inoculums severely affected PCs formation and GSTs activity at substantial magnitude, required for effective conjugation, chelation and subsequent removal of As. On the other hand, accumulation of PCs was much higher and GSTs level was normal in *GSH-overproducing mutant* under As exposure [Talukdar D, unpublished]. Significant high GSH availability powered by elevated GR activity possibly led to rise in PCs level in *GSH-overproducing mutant* during As + inoculums which might facilitate better metalloloid-PC complex and subsequent sequestration of As in the vacuoles. A whole genome transcriptomic analysis in *Crambe abyssinica*, a member of Brassicaceae, pointed out those PCs, GSTs, DHAR, glutaredoxins and GR are intimately involved in As tolerance [68]. GSTs activity in *asfL-1* shoot was constitutively high [42] and PCs level was normal in this mutant during As + inoculums treatment [Talukdar D, unpublished]. Normal PCs level coupled with high GR and GSTs presumably ensured better conjugation and sequestration of As in *asfL-1* mutant through better coordination of GSH-mediated antioxidant defense during As + inoculums-treated mutant plants. Present results clearly pointed out that GSH-dependent antioxidant defense is key for differential behavior between *gshL-1* and *GSH-overproducing* as well as *asfL-1* mutant during combined exposure to As and fungal inoculums. Presumably, high GSH-redox inhibited As-toxicity through up-regulation of antioxidant defense in one hand and efficiently removed As through PCs and/or GSTs in other hand, nullifying fungal infection in tolerant genotypes.

An opposite trend was observed in BioL-212, in which a decrease of PAL activity was associated with an increase in APX and DHAR activities but a concomitant lowering in GR and POX activities. High DHAR activity ensured efficient regeneration of AsA, substrate used by APX as exclusive co-factor during scavenging of H₂O₂ within AsA-GSH cycle [8,18,21,42,46]. However, it seems likely that AsA-dependent antioxidant defense (APX, DHAR and AsA-redox) was not able to prevent *Fusarium* wilting in BioL-212 shoot. This situation got worse when As was added in pot soil. Rise in DHAR activity greatly unbalanced GSH-redox due to continuous consumption of GSH and concomitant generation of GSSG. Moreover, decrease of GR and POX activities severely impeded GSH recycling, resulting in very low GSH-redox and significant rise in H₂O₂ level. Depleted GSH level might also be responsible for lower amount of PCs in BioL-212 exposed to As [Talukdar D, unpublished], affecting efficient removal of As and its detoxification. Despite high APX activity, H₂O₂ content exceeded the level measured as critical for plant growth in inoculated soil and became toxic

to BioL-212, as evidenced by rising MDA content, index of membrane lipid peroxidation in shoots. Excess H₂O₂ have been reported to benefit infection by different pathogens, which may even be able to produce ROS themselves or stimulate the host to do so, thereby, facilitating pathogenic invasion through collapse of peroxisomal antioxidant system and destruction of membrane lipids [62, 69]. Present results, thus, clearly indicated that As exposure escalated wilt infection in BioL-212 and *gshL-1* shoots by a) negatively interfering with PAL activity, b) reducing capability of GR and POX, c) crippling GSH-mediated antioxidant defense through low GSH-redox and presumably, low PCs, and d) enhancing ROS-induced oxidative damage. However, to what extent excess ROS favored pathogen invasion under metalloloid stress, further study is needed to ascertain it.

Conclusions

For the first time, *Fusarium* wilt tolerance was tested in grass pea under As exposure. The results clearly indicated that sensitivity to fungal pathogen is not exclusively dependent on phenylpropanoid metabolism; rather it was intimately associated with GSH-mediated antioxidant defense and its redox state. As exposure jeopardized the PAL activity in three out of four genotypes, yet *asfL-1* mutant uniquely exhibited remarkable tolerance to wilting and oxidative damage. On the other hand, *gshL-1* mutant showed wilting even when PAL activity was normal in inoculums-treated soil. Results revealed that a particular level of H₂O₂ is a critical control point for determining tolerance and sensitivity of grass pea genotypes, and GR and POX enzymes played more vital roles compared to PAL. The differential response of antioxidant defense in inoculums-treated soil and As + inoculums-treated condition revealed pronounced effect of toxic metalloloid As on plant growth of susceptible genotypes in favoring *Fusarium* infection through excess generation of ROS. ROS generation was regulated in tolerant genotypes, maintaining an environment favorable to plant growth. The study provided valuable inputs regarding vulnerability of crop plants under combined exposure to both biotic and abiotic stress factors, which can be used in future breeding to design effective ameliorative strategy for multiple stress tolerant crops.

Competing interests

The author declares no competing interests.

Author's contribution

Sole author DT designed and executed the experiment, prepared the manuscript, and finally approved the revised manuscript.

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