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Increasing *Glycine max* L. Tolerance to Arsenic Stress through Exogenous Aspirin and Tiron

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ABSTRACT

Arsenic (As), a non-essential metalloid in the environment and severely toxic to all the living organisms exist mainly in two inorganic forms arsenate and arsenite. Availability of it in the soil, above the permissible limit, results deleterious impacts on growth and metabolism of most of the plants chiefly via slowing down the cell division and elongation, accumulation of biomass, increased formation of reactive oxygen species (ROS) and alteration in antioxidant defense system. Therefore, to investigate the deleterious impacts of As exposure and its effective amelioration applying aspirin and tiron, seeds of Glycine max L. were subjected to 0, 10 and 100 µM As (sodium arsenite was used as a source of it) and/ or in combination with aspirin (0.1 mM) and tiron (10 mM) separately, for five consecutive days, and were then analyzed. Generated data revealed that increased concentration of As significantly inhibited seed germination, radicle length and biomass (both fresh and dry mass) accumulation, while enhanced the content of reactive oxygen species in five days old Glycine max L. seedlings. However, exogenous addition of both aspirin or tiron significantly augmented As stress tolerance in the five days old Glycine max L. radicles thereby resulting in enhanced germination percentage, radicle length and biomass accrual along with reduced accumulation of ROS. In conclusion, aspirin and tiron application, more particularly aspirin, were observed to confer tolerance, in different magnitudes, against As-induced oxidative stress by limiting ROS production in Glycine max L.

Keywords: Arsenic, Aspirin, Oxidative stress, Reactive oxygen species, Tiron

INTRODUCTION

Plants are constantly exposed to different environmental stresses including heavy metals/metalloids. Arsenic (As) is the 20th most ubiquitous toxic metalloid present in the environment, with an estimated concentration 20 mg kg^{-1} in the Earth's crust [1]. Its contamination is one of the crucial problems for the environment. Both natural and anthropogenic activities such as mining, semi-conductor manufacturing, land irrigation by As contaminated water, use of fossil fuels and As based pesticides/fertilizers in agriculture, and unplanned waste disposal led to severe contamination of it in the surrounding environment [2].

From the soil, As is primarily taken up *via* root absorption system, therefore, root is the first organ that comes in direct contact to available As and show toxicity symptoms [1]. Once it enters inside the root cells, As binds to sulfhydryl groups of both proteins and enzymes that interfere with the functionality of essential proteins in the cell [2]. Therefore, As causes adverse effects in several metabolic processes within the cells, which includes photosynthesis, respiration, growth regulation, reproduction, etc. [3]. These toxic effects result in inhibited seed germination and plant growth, reduced biomass accumulation and/or increased generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), etc. Over-production of ROS is the most widespread reaction resulted from As-stress, culminating into oxidative stress-imposed irreparable injuries [4]. This can directly damage proteins, amino acids and nucleic acids, cause peroxidation of membrane lipids, and finally terminating into cell death [5,6]. To regulate against oxidative stress plants have antioxidant defense system which includes enzymatic components such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), etc. and non-enzymatic components such as proline, glycine betaine, etc. [6,7].

In the recent past, efforts have been continuously made to restore normal growth and development in plants, by exogenously applying a number of potential molecules, exposed to various abiotic stresses including As [4]. Even

though, roles of aspirin and tiron, in the As-stress mitigation in plants are still to be established completely. Aspirin is well known to function as a growth regulator as it provides stress tolerance to the plants by controlling the ROS production and up-regulating antioxidants [8]. Till this date, it has been exploited to modulate adverse signatures of salinity and drought only [9,10], but its mitigation efficiency against metal stress, particularly As, remains to be obscure completely in any of the plant species.

Another molecule, tiron was shown to award abiotic stress tolerance to plants by lowering ROS production *via* NADPH oxidase activity inhibition [11,12]. Additionally, it was also reputed to serve as one of the direct scavengers of $O_2^{\cdot-}$ and has ability to arrest completely the oxidative condition [11]. Screening of the so far published reports led us to conclude that amelioration properties of this potential molecule are yet to be explored completely against As and many other metal/metalloid exerted stress conditions.

Glycine max L. is one of the most important leguminous plants and are sensitive towards As-exposure and show toxicity symptoms [13]. Alleviation of As-toxicity using ROS inhibitors like aspirin and tiron is still to be resolved completely in any of the plant species. Considering this whole background of responses induced under As-stress, the present research aimed to: 1) Analyze the germination percentage and radicle length, 2) Monitor changes in biomass {fresh mass (FM) and dry mass (DM)} accumulation, 3) Investigate the levels of $O_2^{\cdot-}$ and H_2O_2, 4) Localization of $O_2^{\cdot-}$, 5) Alteration in the activities of antioxidant enzymes (SOD, CAT and APX), and 6) Modulation of As-induced stress responses by exogenous addition of aspirin or tiron.

MATERIALS AND METHODS

Seed collection, treatments and germination assessment

Glycine max L. seeds were procured from the local market. Healthy, disinfected seeds were sorted out and washed initially with 1% (v/v) sodium hypochlorite solution for 5 min following washing (5 times) with MilliQ water (MW) (Millipore, Gradient A-10, USA). Seeds were then subjected to MW (control), 10 and 100 µM As (sodium arsenite was used as a source of As) and/or in combination with aspirin (0.1 mM) or tiron (10 mM) and placed for germination over two layers of filter paper soaked with above mentioned solutions in germination boxes of 26 × 16 × 3 cm size [14]. These boxes were kept in darkness at room temperature (28-30°C) so as the seeds can grow. Respective growth medium was supplied to the germinating seeds, whenever needed. On 5th day of growth, germination percentage was assessed and then the radicles were removed carefully. Change in the length of radicles was measured (mm) with the help of a scale. Remaining radicles were stored in sterile plastic vials at -20°C for further analyses. All the analyses were conducted in five replicates and were repeated twice.

Biomass content

To record FM, ten radicles from each replicates were randomly selected, blotted over Whatman filter paper No. 1 and were weighed using an electronic balance (Sartorious, Sweden). To access change in DM accumulation, the radicles were kept in a hot air oven at 103°C for 48 h and then weighed electronically [14].

Reactive oxygen species

Superoxide anion

Liberation of $O_2^{\cdot-}$ was determined by the method given by Sangeetha [15]. Weighed amounts (0.2 g) of tissues were homogenized in 2 ml of chilled sodium phosphate buffer (0.2 M, pH 7.2) containing 0.001 M Diethyl dithiocarbomate {an inhibitor of superoxide dismutase}. The homogenate was then centrifuged (10,000 rpm, 15 min, at 4°C) and the supernatant thus obtained was used as source of $O_2^{\cdot-}$. Superoxide liberation was estimated by utilizing Nitroblue tetrazolium (NBT) as a detection system. The reduction of NBT by $O_2^{\cdot-}$ was detected by recording blue formazan (reduced NBT) at 540 nm using UV-Vis Spectrophotometer (Lambda 25, Perkin Elmer, USA). The calibration curve for pyrogallol oxidation was obtained by determining the rate of NBT reduction using pyrogallol as an $O_2^{\cdot-}$ source, at 540 nm. The amount of $O_2^{\cdot-}$ liberated was expressed as µmol $O_2^{\cdot-}$ min^{-1} g^{-1} FM.

Localization

Sites of $O_2^{\cdot-}$ production in treated and non-treated *Glycine max* L. radicles were localized by nitroblue tetrazolium (NBT) staining [16].

Hydrogen peroxide

To estimate H_2O_2 content, weighed amounts (0.2 g) of control and treated radicles were extracted with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 min at room temperature [17]. An aliquot (0.75 ml) of supernatant was added to equal volume of 10 mM potassium phosphate buffer (pH 7) and 1.5 ml of potassium iodide (1 M). Now, absorbance of the reaction mixture was read at 390 nm. Content of H_2O_2 was expressed as µmol g^{-1} FM.

Antioxidant enzymes

Activity of SOD was determined by estimating the percent inhibition of pyrogallol auto-oxidation by the enzyme at 420 nm [18]. Activity of CAT was assayed by recording the decomposition of H_2O_2 by the enzyme at 240 nm [19]. APX was estimated by recording the rate of ascorbate oxidation at 290 nm [20].

Statistical analyses

Analysis of variance was performed to monitor impacts of As-exposure and other treatments on studied parameters. Correlations between parameters analysed were checked employing Pearson's correlation coefficient test. Data were also analysed for Duncan's multiple range tests, at $p<0.05$. Data depicted are mean ± SE of five separate observations. Statistical analyses were done using SPSS software (Version 16.0).

RESULTS

Germination percentage

To record the change in germination percentage, seeds of *Glycine max* L. were exposed to two different concentrations of As (10 and 100 µM) alone and in combination with aspirin or tiron. Data clearly exemplifies that the germination percentage of *Glycine max* L. were decreased by 74% in response to 100 µM As (Figure 1). On the other hand, application of aspirin or tiron enhanced germination percentage by 40 and 30%, respectively in 100 µM As-stressed *Glycine max* L. seeds (Figure 1). Accumulated data revealed a growth boosting nature of potential molecules. In this concern, the aspirin treatments were more effective than that of tiron.

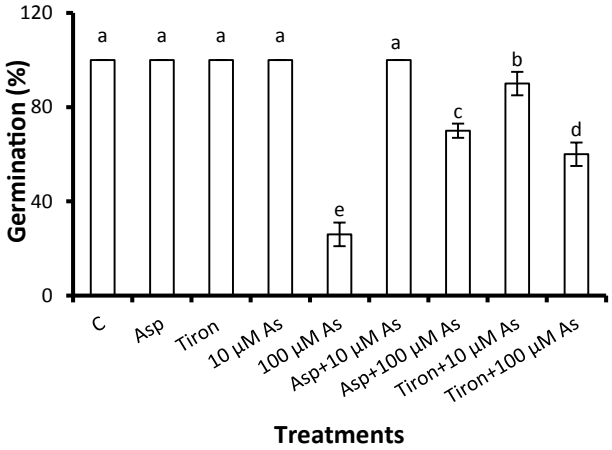

Figure 1: Effects of aspirin or tiron on arsenic induced change in germination percentage in *Glycine max* L. Plotted bars are mean ± SE of five separate observations. Different alphabets show significant difference at the level of $p<0.05$

Radicle length

Radicle lengths of *Glycine max* L. significantly (36-86%) decreased with As addition in five days old *Glycine max* L. compared to MW-treated controls of similar age group (Figure 2). However, coupling of aspirin or tiron with As favored the radicles development (up to 40%) by reducing the inhibitory effects of As in *Glycine max* L. However, between the tested molecules, aspirin was found to be comparatively more efficient than the tiron.

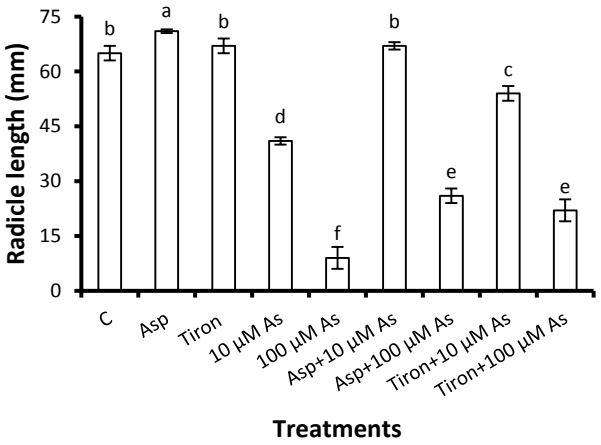

Figure 2: Change in the length of radicles of *Glycine max* L. when exposed to aspirin or tiron with arsenic. Plotted bars are mean ± SE of five replicates. Lowercase letters represents that data are significantly different at the level of $p<0.05$

Biomass accumulation

Biomass content of the growing radicle was monitored following assessment of both FM and DM of the radicles. Data witnessed that accumulations of biomass (FM: 84% and DM: 83%) decreased with the increasing As dose in *Glycine max* L., compared to MW-subjected controls (Figure 3). However, blending of aspirin or tiron with As favored the biomass accumulations by reducing (25-45%) the inhibitory impacts of As in *Glycine max* L. radicles of five days (Figure 3). Of note, aspirin was found to be more efficient than the tiron.

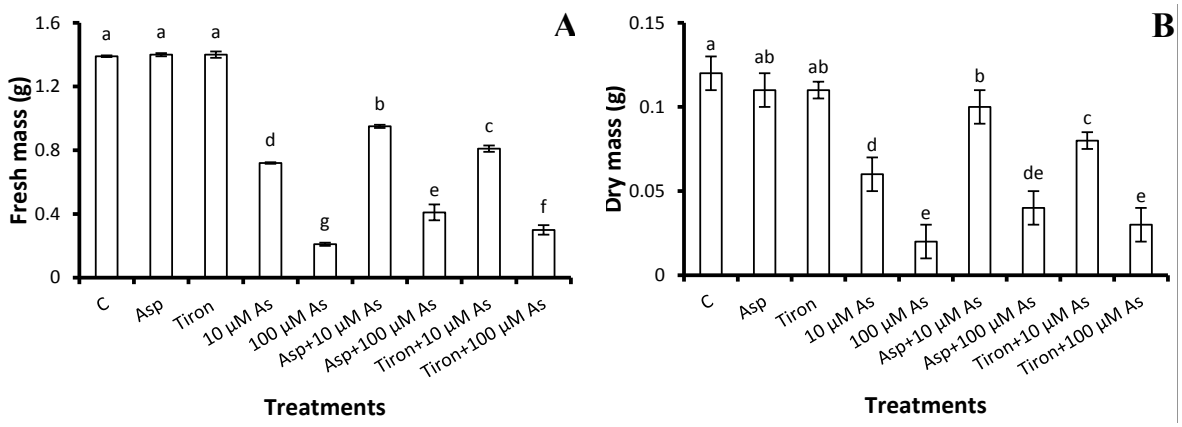

Figure 3: Assessment of difference in fresh mass (A) and dry mass (B) of *Glycine max* L. radicles in response to arsenic, aspirin and/or tiron. Plotted bars are mean ± SE of five separate observations. Alphabets indicate significant difference at $p<0.05$

Reactive oxygen species

Arsenic-exposure caused significant accrual of both the ROS ($O_2^{\cdot-}$: 300-1340% and H_2O_2: 50-244%), compared to MW-grown controls (Figure 4). However, in the aspirin or tiron fortified As solutions, comparatively low levels (35-45%) of ROS were discernible (Figure 4). Data suggested that aspirin played prominent role than that of tiron, to compensate the deleterious impacts of As in *Glycine max* L. radicles of five days.

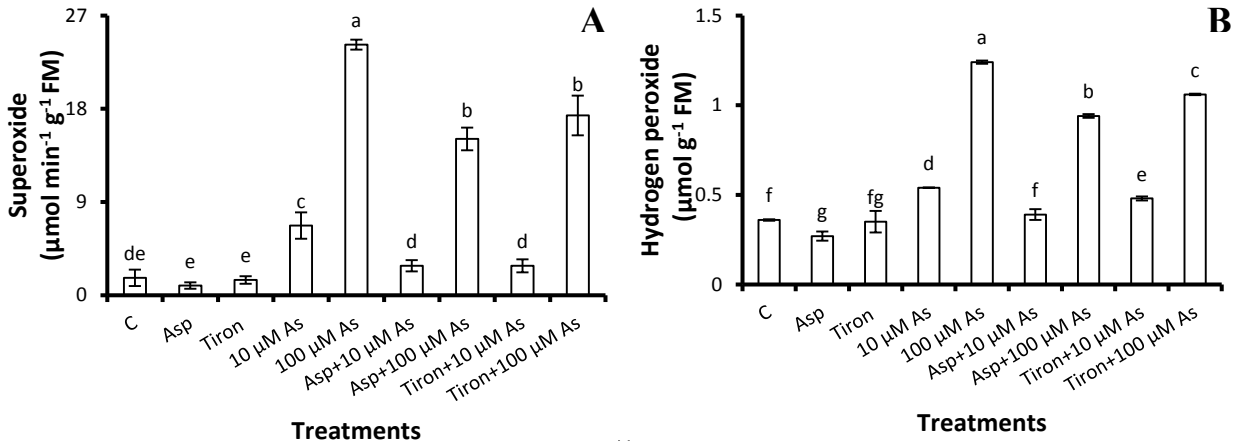

Figure 4: Comparative effects of aspirin or tiron on arsenic-induced superoxide (A) and hydrogen peroxide (B) production in *Glycine max* L. Plotted bars are mean ± SE of five separate observations. Different alphabets show significant difference at the level of $p<0.05$

For the identification of precise locations of $O_2^{\cdot-}$ production, radicles were stained with NBT. Staining revealed that there were more accumulations of blue formazan reaction products in As-subjected radicles, as compared to control (Figure 5). However, application of aspirin or tiron with As resulted in lesser colored patches. Least coloration with NBT was observed in presence of aspirin, thus approving it the more potent treatment in comparison to tiron.

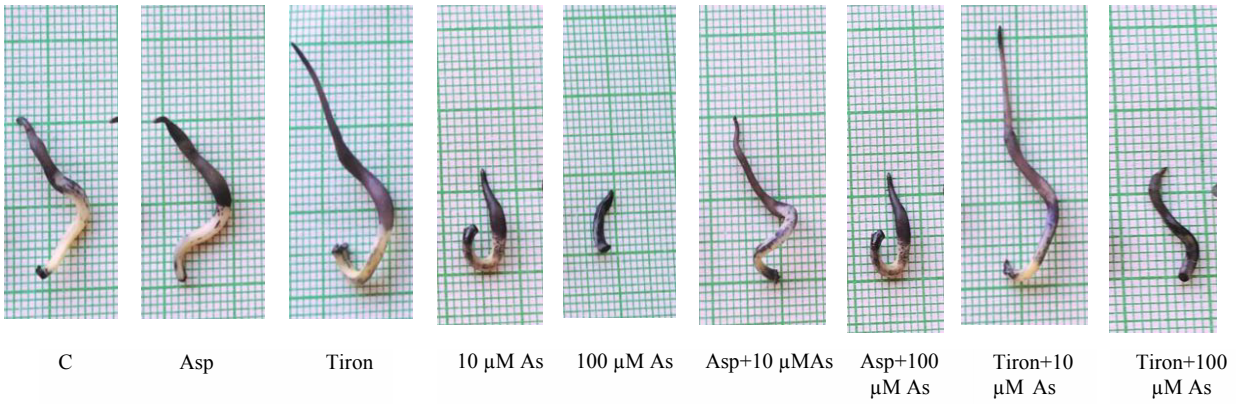

Figure 5: Localization of superoxide generation sites in *Glycine max* L. radicles exposed to various treatments of arsenic, aspirin and tiron

Antioxidant enzymes

Increase in the concentration of As significantly decreased the activities of SOD, CAT and APX (41%, 73% and 68% respectively) in the radicles of *Glycine max* L., but exogenous addition of aspirin or tiron alleviated this decline by 16-65%, under As-stress (Table 1). Highest activities of these enzymes (5.80, 43.90 and 5.47% respectively) were measured in aspirin treatment, in which amount of ROS was least.

Table 1: Comparative effects of aspirin and tiron in the amelioration of arsenic-induced change in the activities of SOD, CAT and APX in *Glycine max* L.

	Superoxide dismutase (min^{-1} mg^{-1} Protein)	**Catalase (nmol min^{-1} mg^{-1} Protein)**	**Ascorbate peroxidase (mmol min^{-1} mg^{-1} Protein)**
Control	217.82^{a} ± 9.38	9.013^{d} ± 0.28	296.5^{a} ± 5.11
Asp	220.44^{a} ± 3.14	12.01^{a} ± 0.14	301.28^{a} ± 4.68
Tiron	219.31^{a} ± 4.49	11.28^{b} ± 0.27	299.64^{a} ± 6.29
10 µM As	197.62^{b} ± 2.37	7.32^{e} ± 0.14	164.67^{c} ± 2.65

100 µM As	102^{e} ± 6.25	2.2^{h} ± 0.19	71.56^{f} ± 3.69
Asp+10 µM As	200.28^{b} ± 3.67	11.56^{ab} ± 0.33	171.98^{b} ± 2.78
Asp+100 µM As	156.39^{c} ± 2.11	5.36^{f} ± 0.15	95.64^{d} ± 2.57
Tiron+10 µM As	198.36^{b} ± 1.49	10.24^{c} ± 0.19	168.59^{bc} ± 1.59
Tiron+100 µM As	139.86^{d} ± 1.97	4.01^{g} ± 0.08	82.37^{e} ± 2.45

DISCUSSION

Presence of As in plants is often accompanied by a variety of metabolic changes. The results of the present study showed that As-treatment resulted in toxicity symptoms like decreased germination percentage, radicle length, biomass content (FM and DM) and activities of antioxidant enzymes, while increased accrual of ROS in Glycine max L. Exogenous addition of aspirin and tiron separately with As in *Glycine max* L. prevented the As-induced oxidative stress up to a large extent and hence enhanced its tolerance.

Exposure of plants to phytotoxic amounts of As induces various physiological and biochemical alterations related with plant growth and development. Seeds of *Glycine max* L. sown in the growing media amended with As exhibited an overall decline in germination percentage and radicle length. It has been proposed that this growth inhibition could partly be due to the loss of cellular turgor, resulting in either decrease in mitotic activity and/ or inhibition of cell elongation [4]. Our results are in coherence with that of Karam et al. [21] in As-exposed *Coriandrum sativum* seedlings. This decrease in growth and development under As-stress was overcome considerably by exogenous addition of aspirin or tiron. Aspirin and tiron are previously reported to restore growth, maintain membrane integrity and hence award tolerance against various abiotic stresses [10,12].

Inhibitory impact of As on *Glycine max* L. seed was estimated by recording the changes in biomass of the growing radicles under the influence of different doses of As. Under As stress, reduced might be due to increased cell permeability and leakage of cellular constituents, that lead to perturbations in energy generation [4]. Our observations on reduced biomass under As-stress substantiated previous reports [3-22], where the authors have noted significant decreases in biomass of *Trigonella foenum* L. and *Lepidium sativum* L. seedlings. However, upon supplementation of As-solution with aspirin or tiron, accumulation of biomass in five days old *Glycine max* L. seedlings was improved significantly (Figure 3). Similar results were also reported in *Cucumis sativus* L. and *Zea mays* L. seedlings under different abiotic stresses [11-23].

To underpin the level of As-induced oxidative stress, ROS ($O_2^{\cdot-}$ and H_2O_2) content was measured in five days old seedlings of *Glycine max* L. Many authors have reported that As-exposure stimulated the formation of ROS in seeds or seedlings leading to oxidative stress [13-23]. Of note, our experimental results also indicated that amount of ROS increased gradually with increased As concentrations (Figure 4). Data from the present study indicates that the As-induced oxidative damage in *Glycine max* L. is reduced by aspirin or tiron supplementation up to a large extent by limiting (35-45%) the ROS accumulation. Aspirin decreases accumulation of ROS either by directly scavenging it or by activating antioxidant defense system [10]. While tiron directly inhibits NADPH oxidase activity, hence hinders with $O_2^{\cdot-}$ production [12].

Increase in the activities of antioxidant enzymes is beneficial for enhancing tolerance against abiotic stress. In the present study, aspirin and tiron improved the antioxidant system that protects *Glycine max* L. from the As-induced oxidative stress. Activities of SOD, CAT and APX were declined in *Glycine max* L. when exposed to increasing concentrations of As (Table 1). This reduction in the activities indicated that these antioxidants were unable to completely detoxify the ROS produced in response to As [4]. Alterations in the activities of antioxidant enzymes in relation to exogenous aspirin or tiron have been observed in *Solanum bulbocastanum* and *Zea mays* L., respectively [10,11]. Both aspirin and tiron were shown to activate or enhance the activities of antioxidant enzymes.

CONCLUSION

The overall results recorded in the current study indicate that As-exposure exerts reduced germination percentage, growth, biomass accrual and activities of antioxidant enzymes in *Glycine max* L., which is evident by increased amounts of ROS ($O_2^{\cdot-}$ and H_2O_2). These toxicity symptoms were overcome considerably when aspirin or tiron was applied with As. Thus, aspirin and tiron increased activities of antioxidant enzymes and served as protective agents against As-stress in *Glycine max* L. Of note, aspirin performed the best in reducing As-induced oxidative stress in

Glycine max L. than the tiron.

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