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□ Herbicide phosphinothricin (PPT) inhibits glutamine synthetase (GS), a key enzyme in nitrogen assimilation, thus causing ammonia accumulation, glutamine depletion and eventually plant death. However, the growth response of *Lotus corniculatus* L. plants immersed in solutions with a broad range of PPT concentrations is biphasic, with pronounced stimulating effect on biomass production at concentrations $\leq 50 \mu\text{M}$ and growth inhibition at higher concentrations. The growth stimulation at low PPT concentrations is a result of activation of chloroplastic isoform GS2, while the growth suppression is caused by inhibition of both cytosolic GS1 and GS2 at higher PPT concentrations. Since the results are obtained in cell-free system (e.g. protein extracts), to which the principles of homeostasis are not applicable, this PPT effect is an unambiguous example of direct stimulation hormesis. A detailed molecular mechanism of concentration-dependent interaction of both PPT and a related GS inhibitor, methionine sulfoximine, with GS holoenzymes is proposed. The mechanism is in concurrence with all experimental and literature data.

Key words: Glufosinate; Glutamine synthetase; Hormesis; Lotus corniculatus L.; Phosphinothricin; Methionine sulfoximine

INTRODUCTION

Hormesis is an effect where a toxic substance (or a harmful physical factor, like radiation) acts like a stimulant at low doses, but it is an inhibitor at high doses. As more precisely defined by Calabrese and Baldwin (2002), hormesis is “*an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis*”. Based on the nature and temporal features of the hormetic response, the authors differentiate direct stimulation hormesis (DSH), and overcompensation stimulation hormesis (OCSH), which is based on modest overcompensation to a disruption in homeostasis (Calabrese and Baldwin 2002).

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In toxicological literature, hormesis has been observed in a variety of model systems, experimental setups and with different agents (Calabrese and Baldwin 2001), including herbicides (Evstigneeva *et al.* 2003; Cedergreen *et al.* 2007; Cedergreen 2008). A comprehensive survey on the effect of nine herbicides, one fungicide and binary mixtures thereof on four plant species showed that 25% to 76% of the dose-response curves for each species had treatments above 105% of the control (Cedergreen *et al.* 2007). However, glyphosate and metsulfuron-methyl, the two herbicides impairing amino acid synthesis, had the highest occurrence of hormetic effect, found in >70% of the analyzed curves (Cedergreen *et al.* 2007). The same two herbicides were shown to induce a real stimulation in biomass growth of $\approx 25\%$ when applied to barley at doses corresponding to 5-10% field rate, whereas other six tested herbicides did not induce consistent hormesis (Cedergreen 2008). Glyphosate blocks the synthesis of aromatic amino acids (Steinrücken and Amrhein 1980), while metsulfuron-methyl inhibits acetolactate synthase (Ray 1984) and thus the biosynthesis of branched amino acids. If the hormetic response is induced or enhanced by some mechanism related to amino acid synthesis, as suggested by Cedergreen *et al.* (2007), then herbicides such as phosphinothricin (PPT), or methionine sulfoximine (MSO), which block a key step in the synthesis of all amino acids, should produce a notable hormetic effect. Indeed, field tests have demonstrated that treatments of plantings with low PPT or MSO concentrations increase the yield of several crops (Evstigneeva *et al.* 2003).

L-phosphinothricin (2-amino-4-(hydroxymethylphosphinyl) butanoic acid) is a naturally occurring glutamate analogue, produced by *Streptomyces viridochromogenes* and *S. hygroscopicus* as a tripeptide L-phosphinothricyl-L-alanyl-L-alanine (Bayer *et al.* 1972; Hoerlein 1994), from which it is released in the target species by intracellular peptidases. PPT is a non-selective postemergence contact herbicide, marketed worldwide either as synthetic racemic mixture or as natural tripeptide, under various trade names (Basta[®], Liberty[®], Hoe 39866, Bialophos, Buster[®], Rely, Finale or Challenge). Glufosinate, the synthetic PPT used in Basta[®] formulation, is a racemic mixture of active L-PPT and inactive D-PPT forms. PPT inhibits glutamine synthetase (GS; EC 6.3.1.2), a key enzyme in nitrogen metabolism that catalyses the formation of glutamine from glutamate and ammonia (Eisenberg *et al.* 2000). PPT mimics the tetrahedral transition state in the enzyme's active site (Eisenberg *et al.* 2000; Unno *et al.* 2006), where it gets phosphorylated to phosphinothricin-phosphate (PPT-P) and remains bound irreversibly (Manderscheid and Wild 1986; Logusch *et al.* 1991; Forlani *et al.* 2006). Structurally related methionine sulfoximine is the first discovered GS inhibitor (Ronzio and Meister 1968). Just like with PPT, the inhibition by MSO is an irreversible process that takes place in the presence of ATP and Mg^{2+} or Mn^{2+} , where MSO is

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phosphorylated and strongly bound to the GS active site as MSO-P (Ronzio and Meister 1968; Rowe *et al.* 1969). Inhibition of GS results in a rapid build-up of photorespiratory ammonia and a concomitant depletion of glutamine and several other amino acids in the plant (Tachibana *et al.* 1986a, b; Hoerlein 1994). These effects are accompanied by a rapid decline of photosynthetic CO₂-fixation and Rubisco inhibition, followed by chlorosis, desiccation and death (Hoerlein 1994; Evstigneeva *et al.* 2003).

Eukaryotic GS proteins have been considered as octamers in a number of studies (Eisenberg *et al.* 2000), but recent crystallographic evidence proposed their decameric composition (Unno *et al.* 2006). Higher plants typically express one chloroplastic (GS2) and one or more cytosolic (GS1) isoforms (Hirel and Gadal 1980; Lam *et al.* 1996). GS2 is predominant in leaves, with a primary role in reassimilation of photorespiratory ammonia (Wallsgrave *et al.* 1987). GS1 isoforms can be expressed throughout the plant, but primarily in nonphotosynthetic tissues, being involved either in primary N assimilation or N remobilization (Muhitch 2003).

In this paper, we show that PPT causes a hormetic effect on biomass production in *Lotus corniculatus* L. (Bird's foot trefoil), a perennial forage legume. The growth stimulation at low PPT concentrations is a result of GS2 activation, while the inhibition of growth and eventually death are caused by inhibition of both GS1 and GS2 at higher PPT concentrations. Since the results with whole plants correlate well with those from a cell-free system (e.g. protein extracts), to which the principles of homeostasis are not applicable, the proposed mechanism of PPT-caused hormesis appears to be by direct stimulation.

MATERIALS AND METHODS

Plant material and culture conditions

L. corniculatus L. cv. Bokor seedlings (Mijatović *et al.* 1986) were introduced into tissue culture as described by Nikolić *et al.* (1997). The rootless plantlets (shoots) were grown *in vitro*, in 360 ml jars containing 60 ml MS medium (Murashige and Skoog 1962) with 3% sucrose and 0.7% agar adjusted to pH 5.8. The cultures were grown under a 16 h photoperiod, under photon flux rate of $\approx 40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the level of the plants, at 25 ± 2 °C and $\approx 70\%$ relative humidity within the jars. The 3-weeks old shoots were used either for culture maintenance by shoot sub-culture, for Basta[®] treatment, for chloroplast isolation or for protein isolation.

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Chloroplast isolation

To obtain a chloroplast enriched fraction, 6 g of fresh leaves was homogenized with a blender in 30 ml ice-cold buffer containing 50 mM Hepes pH 7.5, 5 mM DTT, 330 mM sorbitol, 1 mM EDTA, 1 mM MgCl₂ and 1% soluble polyvinylpyrrolidone, for 30 s. The homogenate was filtered through one layer of cotton wool and five layers of gauze and centrifuged for 5 min at 3000 g. Microscopic inspection of the obtained pellet revealed the presence of intact chloroplasts as well as other smaller organelles and cell wall debris, but not intact cells, and thus no cytosol.

Protein extraction, separation and GS assay

Soluble proteins were extracted either from 1 g of fresh leaves or from chloroplast pellet by grinding in liquid nitrogen followed by homogenization in protein extraction buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 1.5% (w/v) insoluble polyvinylpyrrolidone, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 20% v/v glycerol). The homogenate was centrifuged for 15 min at 15,000 g at 4 °C. The obtained supernatants were frozen in liquid nitrogen and kept at -70 °C until use. The protein concentration was quantified by Bradford method (Bradford 1976), with BSA as standard. Prior to electrophoresis, the samples were diluted with the extraction buffer (polyvinylpyrrolidone omitted) to a final concentration of 2 mg·ml⁻¹. Proteins were separated on discontinuous nondenaturing 7% polyacrylamide gels, using Hoefer SE600, at 150 V for 20-22 h at 4 °C. The gels with separated GS isoforms were incubated in GS assay buffer containing 20 mM Na-glutamate, 20 mM ATP, 20 mM NH₄Cl, 20 mM MgCl₂, and 50 mM KCl in 100 mM Tris-HCl pH 8, for 30 min at 35 °C. The GS activity bands were visualized by in-gel phosphate precipitation assay, as described previously (Simonović *et al.* 2004).

Experimental design and PPT application

In order to study the effect of PPT on plant growth and GS activity, the herbicide was applied as Basta® formulation (Bayer Crop Science) to rootless plantlets (experimental setup 1), or as D,L-phosphinothricin (Fluka) to the assay mixtures (2) or protein extracts (3 and 4). In some experiments another GS inhibitor, MSO (D,L-methionine-D,L-sulfoximine from Sigma), was used for comparison.

- (1) To evaluate the effect of low PPT concentrations on biomass production, rootless plantlets were treated with the herbicide. Three-week old shoots grown *in vitro* were weighed, the individual masses were recorded, and then the shoots were immersed for 5 minutes in sterile Basta® formulation serially diluted to contain 1 - 200 µM PPT. Control plants were immersed in sterile distilled water. After the PPT treat-

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ment, the plantlets were returned to fresh MS medium (five plants per jar). The biomass increase was recorded after 15 days. The same protocol with 200 μM PPT was used to study the effect of foliar application of the herbicide on GS activity, in which case the plants were harvested for protein isolation 5 days after the treatment.

- (2) To assess the sensitivity of separated GS isoforms to PPT inhibition, PPT was added to GS assay buffer for incubation of the gels at final concentrations ranging from 100 to 700 μM .
- (3) In experiments addressing the electrophoretic mobility shift (EMS) of GS isoforms caused by inhibitor binding, PPT was added to foliar protein extracts prior to electrophoresis. Protein extracts containing 100 μg of soluble protein in 50 μl volume were mixed with PPT in a range of 1 μM – 10 mM final concentrations and with components required for GS activity in the following final concentrations: 20 mM ATP, 50 mM KCl, 20 mM MgCl_2 and 2 mM EDTA. For comparison, parallel sets of samples were prepared without ATP or with ATP and MSO (1 μM – 1 mM) instead of PPT. The final volume was brought to 100 μl with water. All samples were incubated at room temperature for 20 minutes, and then separated and assayed as described.
- (4) Finally, the question of reversibility of PPT binding to GS was studied using protein extracts with 50 μM PPT, 20 μM ATP and other components as in (3), which were subjected to gel filtration after the incubation step in order to separate proteins from low MW compounds including unbound PPT. The protein samples were loaded to Sephadex G25 columns equilibrated in one of three buffer systems, eluted with the same buffer used for equilibration, and 50 μl fractions were collected and analyzed. The first buffer contained 50 mM Tris-HCl pH 8 and 1 mM EDTA, the second was 50 mM Na-acetate pH 4.5 with 1 M KCl and 0.4 M $(\text{NH}_4)_2\text{SO}_4$, and the third was GS assay buffer that contains substrates.

Data analysis

Densitometric analyses were performed with TotalLab TL120 (Nonlinear Dynamics Ltd. Newcastle, UK) with the following parameters: background was subtracted with the rolling ball method set at 200, bands were hand picked, and gaussians were fitted to peaks with an advanced algorithm. The significance of differences among the treatments was determined by analysis of variance (ANOVA) using STATISTICA 8 (Statsoft, Inc. Tulsa, OK, USA). Means were compared using Fisher's multiple range test at a significance level of $P < 0.05$.

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RESULTS AND DISCUSSION

Low doses of PPT stimulate biomass production in *L. corniculatus*

In the course of defining the optimal PPT concentration for selection of Basta-resistant *L. corniculatus* plants transformed with *bar* gene (unpublished data), it was noticed that low PPT concentrations actually promote plant growth *in vitro*. To confirm this finding, ten shoots from *in vitro* culture were immersed in Basta solutions containing PPT in a range from 1 to 200 μM and returned to MS medium for additional two weeks. The plants treated with 12.5 and 25 μM PPT were noticeably larger in com-

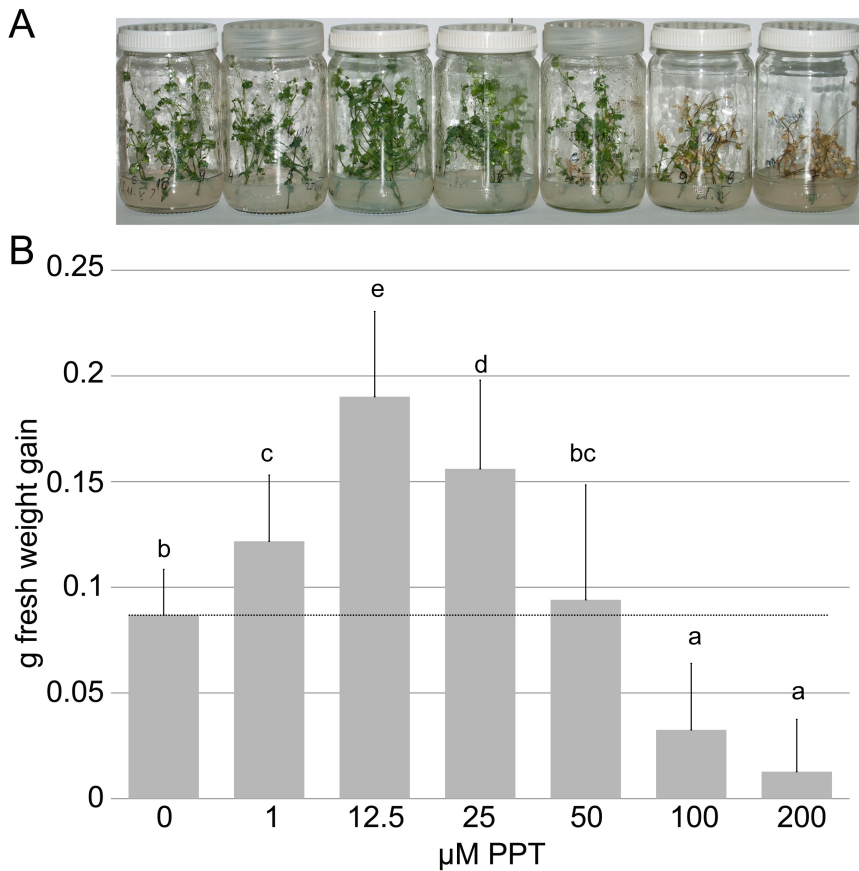


FIGURE 1. Phosphinothricin causes prominent hormesis upon foliar application to *in vitro* grown *L. corniculatus* plantlets. **A** - *L. corniculatus* shoots were immersed in Basta® solutions of indicated effective PPT concentrations or sterile water for 5 min and grown on fresh MS medium for 2 weeks. **B** - The fresh weight of each plant was recorded prior to PPT treatment and after two weeks, and the average biomass production (weight gain) is presented. The values presented are means for two replicates with 5 plants each ($n = 10$), with indicated standard deviations. Statistical difference at the significance level of $P < 0.05$ is designated by different letter above the bars, as calculated by Fisher test.

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parison to the control, while concentrations above 100 μM where lethal (Fig. 1A). The fresh weight gain of *L. corniculatus* shoots treated with PPT showed a biphasic response with stimulatory effect for concentrations $\leq 50 \mu\text{M}$ and toxic effect above 100 μM (Fig. 1B), which is, by definition (Calabrese and Baldwin 2002), a hormetic effect. Even though the magnitudes of published cases of hormetic stimulatory responses are generally 30 - 60% greater than controls (Calabrese and Baldwin 2002), in the case of *L. corniculatus* the amplitude of stimulatory effect at 12.5 μM PPT, was as high as 119% for fresh weight gain and 62% for total fresh weight. In a thorough study on the effect of nine herbicides, one fungicide and their binary mixtures on four plant species, Cedergreen *et al.* (2007) reported that dry weight at harvest showed a higher frequency and a larger hormetic response compared to relative growth rates. This finding only strengthens the relevance of our results, where hormesis was clear and prominent even with fresh weight gain as a growth parameter. The width of the stimulatory response was within 50-fold PPT concentration range (from 1 to 50 μM), and thus concordant with the literature data on hormetic stimulation, which are typically (in 90% cases) in the 5- to 100-fold dosage range (Calabrese and Baldwin 2002).

To our best knowledge, this is the first time that the effect of PPT on plant growth was shown to be biphasic and thus defined as hormetic. However, several earlier reports indicated that low PPT concentrations could stimulate plant growth and morphogenesis. It was shown that application of PPT in low concentrations to several field crops can increase the yield (Evsstigneeva *et al.* 2003). Subtoxic concentrations of PPT facilitate plant regeneration from *Gladiolus* calli (Kamo and van Eck 1997), stimulate somatic embryogenesis in grape (Hébert-Soulé *et al.* 1995), and promote shoot regeneration from hairy roots of *Antirrhinum majus* L. (Hoshino and Mii 1998). Rather than to investigate the effect of different PPT concentrations on other morphometric or biochemical parameters of the *in vitro* *L. corniculatus* cultures, we decided to explore the effects of PPT on its target – glutamine synthetase.

Electrophoretic profile of GS isoenzymes from *L. corniculatus*

Analysis of soluble proteins from *L. corniculatus* shoots by native PAGE followed by in-gel assay (Simonović *et al.* 2004) revealed two GS activity bands in the expected MW range for plant octameric enzymes (which are invariably $\geq 300 \text{ kDa}$) and one additional high mobility band (Fig. 2A). The GS activity that is also present in chloroplastic fraction is, by definition, GS2 isoform, while the other high MW band is cytosolic GS1. There are no literature data describing GS isoforms in *L. corniculatus*, but in a closely related model legume, *L. japonicus*, GS2 and a single GS1 isoforms were detected (Harrison *et al.* 2003; Betti *et al.* 2006). In addition to GS2 and GS1 octamers, Betti *et al.* (2006) also detected one tetrameric GS pro-

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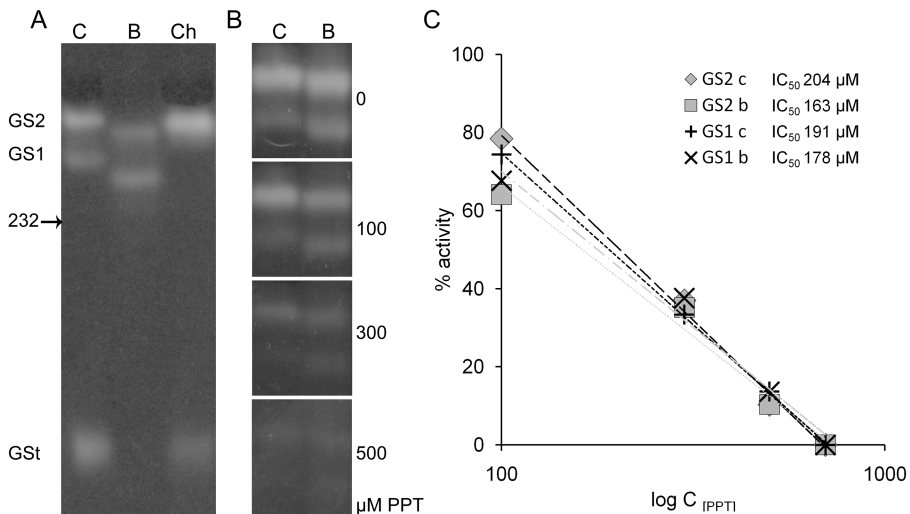


FIGURE 2. Zymogram of GS isoforms from *L. corniculatus*. **A** – Soluble proteins were extracted from shoots (C), Basta-treated shoots (B) or isolated chloroplasts from control plants (Ch), separated by native PAGE and assayed for GS activity. Indicated are chloroplastic isoform GS2, cytosolic isoform GS1 and a fast migrating GSt activity that is probably a tetrameric GS2 dissociation product. The position of bovine liver catalase (MW = 232 KDa) is also indicated. **B** – Inhibition of GS activity in protein extracts from shoots (C) or Basta-treated shoots (B) by increasing concentrations of PPT added to the GS assay buffer for the incubation of the gels. **C** – Activity of the GS isoforms from Fig. 2B was densitometrically measured and the concentrations causing 50% inhibition (IC₅₀) of in gel activity were estimated by utilizing the linear regression equation of the % activity values plotted against the logarithm of inhibitor concentration.

tein in Western blots of native PAGE gels. The tetramer represented dissociated GS2 subunits with no activity, so it is unclear whether *L. japonicus* GS2 tetramers are physiologically significant or an isolation artifact. Since the fast migrating GS activity in our zymograms is present in the chloroplast sample, it is likely also a product of GS2 dissociation, presumably a tetramer (and thus labeled as GSt). Unlike the *L. japonicus* tetramer, GSt from *L. corniculatus* is catalytically active. Catalytically active GS2 tetramers were also found in sugar beet leaves (Mäck and Tischner 1994; Brechlin *et al.* 2000), where GS2 octamer/tetramer ratio changed during the leaf ontogeny, so in mature leaves 80% of total GS2 activity was due the activity of the tetrameric form. These findings suggest that GS tetramers are physiologically relevant forms rather than just artifacts. In sugar beet hairy roots, both GS1 and GS2 were active in the octameric and tetrameric state (Mack 1998).

Interestingly, both GS1 and GS2 from Basta-treated plants displayed an electrophoretic mobility shift in comparison to the enzymes from control plants (Fig. 2A), probably due to an increased charge and/or conformational change caused by PPT binding. GSt activity was never observed in extracts of plants treated with Basta®, so PPT either promotes

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association of the tetramers into octamers or completely inhibits the activity of tetramers.

When PPT is added to the GS assay buffer in concentrations above 100 μM , it inhibits GS1 and GS2 from both control and Basta-treated plants (Fig. 2B). When applied at 700 μM concentration, PPT completely inhibits all GS activity (data not shown). The estimated IC_{50} values for GS activity bands (Fig. 2C) from Basta[®] treated plants were slightly lower (163 μM for GS2 and 178 μM for GS1) compared to GS activity bands from control plants (204 μM for GS2 and 191 μM for GS1), possibly caused by *in vivo* bound PPT to GS from Basta[®] treated plants.

PPT binding increases GS1 and GS2 electrophoretic mobility and activates GS2

Since the treatment of plants with Basta[®] causes EMS of extracted GS (Fig. 2A), a series of experiments were set to demonstrate that the shift is a direct consequence of PPT-GS interaction and to elucidate whether some factors present in tissues are required for the interaction *in vitro*. Addition of PPT only, in a broad range of concentrations, to protein extracts (prior to electrophoresis and activity staining) had no effect whatsoever on GS activity or mobility (Fig. 3A). However, when the extracts were incubated with PPT in the presence of ATP, a concentration dependant EMS of both GS2 and GS1 was observed in range of 1-30 μM PPT (Fig. 3B), where the shift magnitude reaches a plateau (Fig. 3C). The observed EMS can be a result of increased charge of the PPT-enzyme complex and/or altered GS conformation. The fact that EMS was observed on GS zymograms and only in the presence of ATP has three implications: first, PPT likely binds to the active sites of the enzyme, not to allosteric sites; second, the PPT-GS interaction is irreversible at low PPT concentrations, and third, PPT binds irreversibly only to some subunits of the octamer. Namely, the requirement for ATP for the EMS to occur indicates that PPT is phosphorylated by the enzyme, which can happen only at the active site. It is well established, based on kinetic and crystallographic evidence, that phosphorylation of PPT (just like with other GS inhibitors) is required for its binding to both eukaryotic and bacterial GS enzymes, and that the binding is irreversible but non-covalent (Manderscheid and Wild 1986; Logusch *et al.* 1991; Abell and Villafranca 1991; Eisenberg *et al.* 2000; Gill and Eisenberg 2001; Forlani *et al.* 2006; Berlicki 2008). Our results, based on electrophoretic mobility shift assay (EMSA), are unique as approach in the study of GS-inhibitors interactions, but only support the published kinetic and crystallographic data. EMSA is commonly used to study DNA- and RNA-protein interactions (Hellman and Fried 2007), but is rarely observable with small ligand-protein interactions, unless the ligand is modified with a bulky attachment (Funabashi *et al.* 2007). Since electrophoresis removes free PPT present

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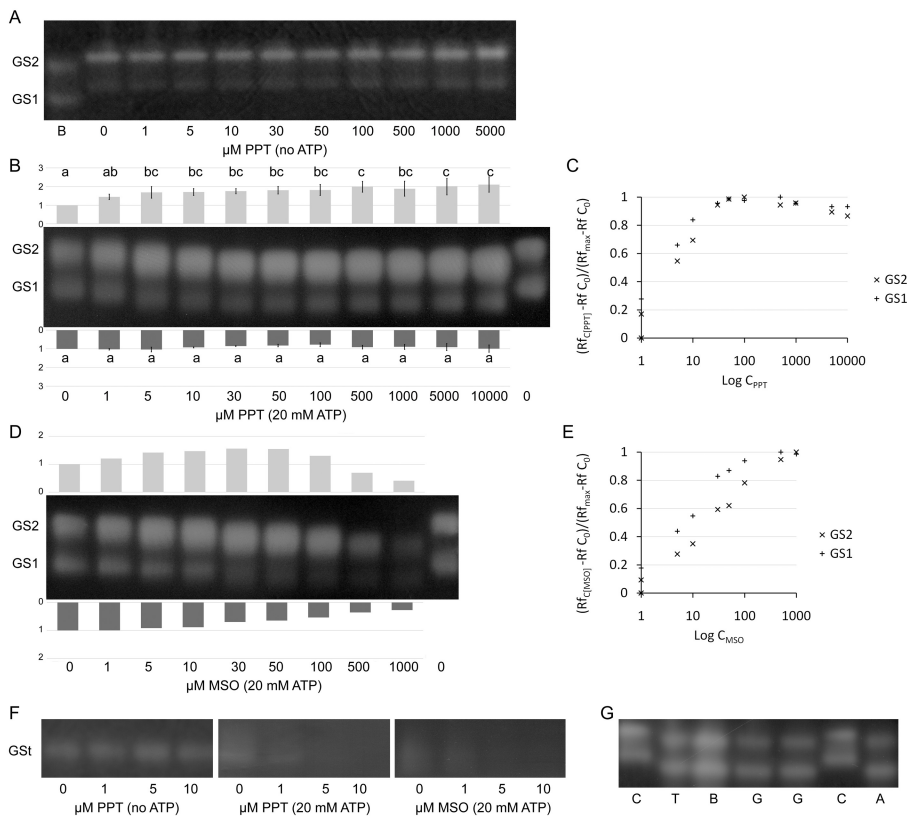


FIGURE 3. Effect of PPT and MSO added to crude protein extracts in micromolar range prior to electrophoretic separation. **A** – Addition of PPT to protein extracts in a range of concentrations from 1 to 5000 μM had no effect on GS activity or mobility in the absence of ATP (**B** – protein extract from Basta-treated plant loaded for mobility comparison). **B** – Addition of various concentrations of PPT in the presence of 20 mM ATP to protein extracts causes an increase of electrophoretic mobility of both GS1 and GS2, with concomitant stimulation of GS2 catalytic activity. The experiment was performed three times; presented are means of GS activity in arbitrary densitometric units, with bars indicating SD. Statistical difference at the significance level of $P < 0.05$ is designated by different letter above the bars, as calculated by Fisher test. **C** – Relative mobility shift of GS isoforms, as plotted against log PPT concentration, indicates that the shift magnitude reaches a plateau at 30-50 μM PPT. The shift is expressed as $(Rf_{C[\text{PPT}]} - Rf_{C0}) / (Rf_{\text{max}} - Rf_{C0})$ ratio, where $Rf_{C[\text{PPT}]}$ is retention factor of a GS isoform at a certain PPT concentration, Rf_{C0} is Rf of GS isoform at 0 PPT concentration, and Rf_{max} is maximum Rf measured. This transformation gives a value of 0 for isoforms in the sample with no PPT and 1 for isoforms in the sample with maximum mobility shift. For each band, the densitometric peak was used for Rf value. **D** – Binding of MSO in the presence of 20 mM ATP to GS proteins in the crude extract also causes electrophoretic shift of both GS1 and GS2 isoforms, with a biphasic concentration effect on GS2 activity and progressive inhibition of GS1 activity. **E** – Relative mobility shift of GS isoforms with increasing MSO concentration (calculated as in Fig. 3C) does not reach a plateau, because both isoforms are eventually completely inhibited. **F** – Very low concentrations ($< 5 \mu\text{M}$) of either PPT or MSO are sufficient to completely inhibit activity of tetrameric GS isoforms, possibly by stimulating their association into octamers. ATP is required for this effect. **G** - Crude protein extracts were incubated with 50 μM PPT and 20 mM ATP, subjected to gel filtration with different buffer systems and analyzed for GS activity and electrophoretic mobility. The following gel filtration fractions were used: C – control extracts not treated with PPT, T - fraction equilibrated in 50 mM Tris-HCl pH 8, 1 mM EDTA, B - Basta treated plant extract used as mobility reference, G - fractions equilibrated in GS buffer with substrates, A - fraction equilibrated in 50 mM Na-acetate pH 4.5 with 1 M KCl and 0,4 M $(\text{NH}_4)_2\text{SO}_4$.

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in the samples, and consequently promotes dissociation of any reversibly bound PPT (if present) due to equilibrium change, the observed EMS is probably caused by irreversible or very strong PPT-GS binding. Finally, the fact that PPT-bound GS holoenzymes have not only significant but increased catalytic activity means that PPT is irreversibly bound only to some but not all subunits of the octamer.

It should be mentioned that PPT exhibits the kinetic properties of a slow tight-binding inhibitor, with the rate-limiting step being its binding, because the enzyme has to bind a molecule that has a “transition-state” geometry rather than a ground-state substrate structure (Abell and Villafranca 1991). Nevertheless, the inhibition reaction completes within milliseconds or seconds, depending on PPT concentration (Abell and Villafranca 1991), so the incubation time of protein extracts with PPT and ATP of 20 min used in our experiments should be more than sufficient for the reaction to complete.

Even though the discussed EMS is sufficient evidence *per se* that PPT and MSO binding to some or all GS subunits is irreversible, the strength of the GS-PPT interactions was additionally documented by gel filtration experiments (Fig. 3G). Protein extracts were incubated with 50 μ M PPT and 20 μ M ATP and then subjected to Sephadex gel filtration in different buffer systems. The first buffer (50 mM Tris-HCl pH 8 and 1 mM EDTA) was used just to remove all free PPT from the samples, the acidic buffer (50 mM Na-acetate pH 4.5 with 1 M KCl and 0.4 M $(\text{NH}_4)_2\text{SO}_4$) was used in an attempt to remove the acid-labile phosphate from PPT-P and thus promote its dissociation from the enzyme, and the third was GS assay buffer that contains substrates, which was used to maximize the competition for the active sites. Colanduoni and Villafranca (1986) showed that the acidic buffer can reactivate bacterial GS inhibited by PPT, but in our study none of the buffer systems was able to destabilize or prevent GS-PPT binding and the consequent EMS (Fig. 3G).

Irreversible binding of PPT to GS leads to statistically significant activation of the plastidic GS2 isoform, whereas the activity of cytosolic GS1 remains unchanged (Fig. 3B). PPT, PPT-P, MSO, MSO-P and some other derivatives thereof, were reported to activate GS from *Phaseolus vulgaris* and *T. aestivum* when applied in micromolar concentrations, while millimolar concentrations were inhibiting (Evstigneeva *et al.* 2003 and references therein). Migge *et al.* (2000) showed that leaf-specific overexpression of GS2 stimulates the growth of transgenic tobacco seedlings. The increased GS2 activity was correlated with a decrease in the leaf ammonium pool, increase in the levels of free glutamate and glutamine and increase of biomass production of the transgenic tobacco compared to untransformed seedlings (Migge *et al.* 2000). On the contrary, experiments with antisense expression of GS1 in *Lotus japonicus* nodules (Harrison *et al.* 2003), and overexpression of GS1 in *L. japonicus* roots

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(Limami *et al.* 1999) showed that GS1 activity is negatively correlated with the biomass production. Our results, supported by the literature data, suggest that the stimulatory effect of low PPT concentrations on biomass production in *L. corniculatus* is a direct consequence of GS2 activation. Performing experiments on protein extracts makes it unlikely that the observed hormetic effect is based on overcompensation at the organismal or cellular level. Knowing that one of the key conceptual features of OCSH is the disruption of homeostasis coupled to an overcompensation response (Calabrese and Baldwin 2002), and that homeostasis is a property of a system, it appears that the OCSH mechanism is not applicable to our data. Therefore, the effect of PPT on *L. corniculatus* is apparently direct stimulation hormesis.

Addition of MSO in a micromolar range to crude protein extracts had no effect in the absence of ATP (data not shown), while in the presence of ATP, MSO caused electrophoretic shift of both GS1 and GS2 (Fig. 3D). However, the effects of PPT and MSO on GS activities are different. MSO stimulates GS2 activity up to a concentration of 30-50 μM , but higher concentrations are inhibitory. Since the highest mobility shift of both GS1 and GS2 corresponds to lowest activity of the enzymes, with log-linear concentration dependence of the shift (Fig. 3E), it can be concluded that MSO, unlike PPT, binds to all GS subunits irreversibly. Both MSO and PPT cause complete disappearance of GS activity at concentrations as low as 5 μM (Fig. 3F), but it is unclear whether this effect is a result of GS inhibition or/and re-association of the GS tetramers into GS2 octamers. Maurizi and Ginsburg (1982) showed that MSO binding in the presence of ATP promotes re-association of *E. coli* GS subunits into dodecamers, so it is possible that a similar effect also occurs with plant enzyme.

Proposed mechanism of phosphinothricin hormesis in *L. corniculatus*

The proposed mechanism of PPT direct stimulation hormesis (Fig. 4) is based on the following experimental evidence:

- (1) PPT at concentrations $\leq 50 \mu\text{M}$ enhances the biomass production, while the concentrations $> 50 \mu\text{M}$ are toxic or lethal (Fig. 1).
- (2) PPT binding to GS in planta (Fig. 2A) or in vitro (Fig. 3B) causes an increase in electrophoretic mobility of the holoenzyme.
- (3) The PPT effects on GS activity and mobility are ATP-dependent (Fig. 3A and B), indicating that PPT binds to the active sites in the form of PPT-P, and not to some allosteric sites.
- (4) PPT-GS interaction is irreversible at low PPT concentrations, since bound PPT cannot be removed neither by electrophoresis (Fig. 3B) nor by gel filtration in different buffer systems that should promote inhibitor dissociation or inhibitor-substrate competition (Fig. 3G).

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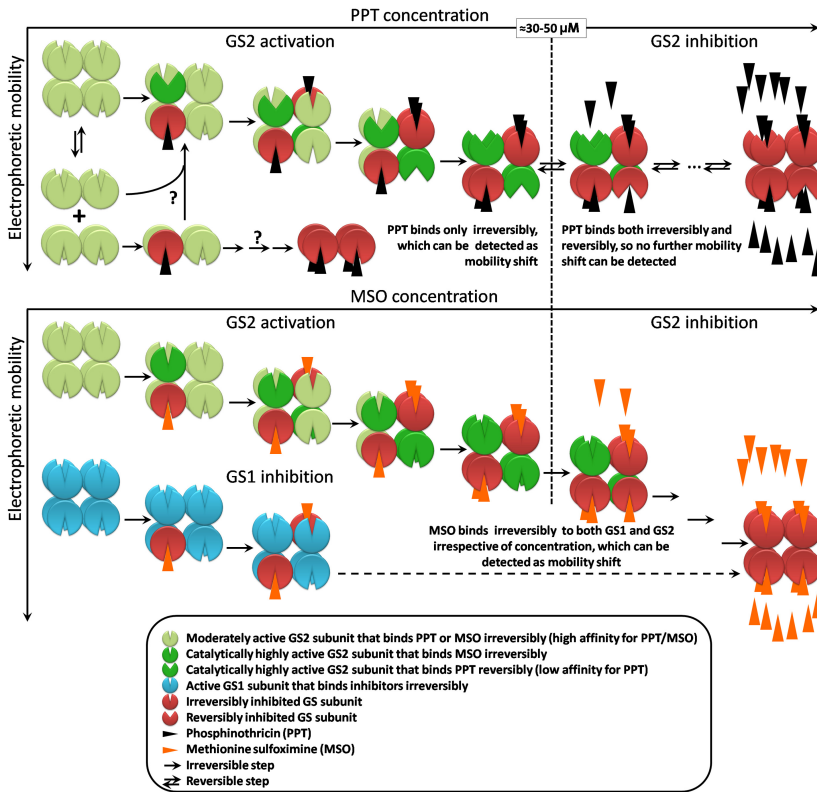


FIGURE 4. A hypothetical mechanism of PPT direct stimulation hormesis at a molecular level. **GS2-PPT interaction.** At low concentrations PPT binds to GS2 subunits irreversibly, causing their inhibition, but concomitantly altering the conformation of another (presumably neighboring) subunit. The subunits with altered conformation have higher catalytic activity, which is the basis of the observed GS2 activation, but also lower affinity for PPT and thus they bind PPT reversibly. The inhibition of one GS2 subunit is overcompensated by increased activity of a neighboring subunit, resulting in holoenzyme net activation. PPT binding causes an increase in electrophoretic mobility of the holoenzymes based on increased charge and/or conformational change. Very low PPT concentrations ($< 5 \mu\text{M}$) completely eliminate GS2 activity, either by their inhibition and/or by promotion of their association into octamers (which would also contribute to GS2 activation). At higher PPT concentrations ($\geq 50 \mu\text{M}$), when the active sites of high PPT-affinity GS2 subunits are saturated with the inhibitor, further increase in PPT concentration leads to progressive inhibition of low-affinity subunits and eventually to complete inhibition of the holoenzyme. **GS1-PPT interaction.** PPT binds to GS1 in a similar, sequential, manner as to GS2, causing the same gradual EMS, but it does not affect the net activity of the octamers. It can be speculated that the inhibition of one GS1 subunit is accompanied with equivalent activation of another subunit, resulting in no net change in activity. Thus, the only difference between GS1-PPT and GS2-PPT interaction is the degree of activation of the neighboring subunits (not shown). **GS2-MSO interaction.** The mechanism of GS2-MSO interaction is similar to GS2-PPT interaction, with a noticeable difference that upon MSO binding to one GS2 subunit, a neighboring subunit retains the same affinity for MSO, even though it is activated. In other words, MSO binds to all GS2 subunits irreversibly. The net increase in GS2 activity at 1-50 μM MSO depends on the ratio of moderately active, highly active and inhibited subunits, while at higher concentrations the inhibited subunits are predominant. MSO likely has the same effect on GS1 (not shown). **GS1-MSO interaction.** Finally, MSO binding to GS1 causes simple sequential inhibition of the subunits with concordant EMS, but with no activating effect on the neighboring subunits.

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- (5) PPT binds irreversibly only to some, but not to all GS subunits, since GS-PPT complexes (e.g. GS proteins that display EMS) retain enzymatic activity and are visible after in-gel assay (Fig. 3B).
- (6) Irreversibly bound PPT stimulates the activity of the GS2 holoenzyme, but does not affect activity of GS1 holoenzyme (Fig. 3B). The highest GS2 activity is found for samples with highest EMS.
- (7) When present in higher concentrations, PPT can bind to all the remaining GS subunits and inhibit the GS activity completely. This type of interaction is reversible, because even holoenzymes that show maximal EMS, and therefore have saturated the irreversible binding sites, are still sensitive to PPT inhibition (Fig. 2B).
- (8) GSt is an active GS2 derivative, since it is present as high mobility band in chloroplast preparations (Fig. 2A), and is likely a tetramer (literature evidence).
- (9) PPT completely eliminates the GSt activity (Fig. 2A and Fig. 3F).

The model also explains the mode of MSO-GS interactions, and it is based on the following facts:

- (1) Binding of MSO to either GS1 or GS2 is irreversible at all concentrations, because MSO added to the protein extracts can completely inhibit the GS activity (Fig. 3D), and the EMS increase is without a plateau (Fig. 3E). The holoenzymes with the highest EMS have the lowest activity.
- (2) ATP is required for the effect of MSO, just as is the case with PPT (data not shown).
- (3) MSO in concentrations up to 50 μM stimulates, but at higher concentrations inhibits GS2 activity (Fig. 3D)
- (4) Inhibition of GS1 in the presence of MSO is progressive and linear (Fig. 3D)
- (5) MSO completely inhibits GSt activity in the presence of ATP (Fig. 3F)

The proposed model is in agreement with all the obtained experimental evidence and literature data, but is still hypothetical, because many suggested steps require independent experimental support. The basis of the mechanism is communication between the subunits or their active sites, which has been documented for *E. coli* GS enzyme (Abell and Villafranca 1991). The model proposes that in the irreversible GS-inhibitor interactions both PPT and MSO are phosphorylated at the active site to PPT-P or MSO-P, based on the ATP requirement, but there is no direct evidence that PPT or MSO do *not* bind to allosteric sites. Furthermore, the proposed stoichiometry and topology of interactions among the inhibited and activated GS subunits is entirely speculative. The model represents GS enzymes as octamers, which is generally accept-

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ed, even though crystallographic evidence suggests that they are decamers (Unno *et al.* 2006). However, the principles underlying the proposed mechanism are applicable to decamers as well. The competition between PPT or MSO and the substrate, glutamate, for the active sites, as well as phosphorylation of the inhibitors are omitted from the illustration for the sake of clarity. The model explains that only activation of GS2 and not GS1 is the basis for the hormetic response observed at the whole plant level (Fig. 1), which is in agreement with the fact that only GS2 activity is positively correlated with biomass production (Limami *et al.* 1999; Migge *et al.* 2000; Harrison *et al.* 2003). We believe that our study would contribute to better understanding of hormetic effects of the herbicides with possible practical application on yield increase in crops (Belz *et al.* 2011).

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ABBREVIATIONS:

DSH - direct stimulation hormesis
 EMS - electrophoretic mobility shift
 EMSA - electrophoretic mobility shift assay
 GS - glutamine synthetase
 GS1 - glutamine synthetase cytosolic isoform
 GS2 - glutamine synthetase chloroplastic isoform
 GSt - glutamine synthetase tetramer
 MS - Murashige and Skoog medium
 MSO - D,L-methionine-D,L-sulfoximine
 MSO-P – methionine sulfoximine phosphate
 OCSH - overcompensation stimulation hormesis
 PPT - D,L-phosphinothricin
 PPT-P - phosphinothricin-phosphate

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