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Growth, ammonia accumulation and glutamine synthetase activity in alfalfa (*Medicago sativa* L.) shoots and cell cultures treated with phosphinothricin

Lori C. Krieg, Mark A. Walker, Tissa Senaratna, and Bryan D. McKersie

Department of Crop Science, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

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Summary. Phosphinothricin is a non-selective herbicide which inhibits glutamine synthetase (EC 6.3.1.2) activity causing an overaccumulation of ammonia in higher plants. Alfalfa (*Medicago sativa* L) shoot tissue and petiole-derived callus exposed to phosphinothricin show 50 and 70% reductions, respectively, in glutamine synthetase activity with a concomitant rise of 10 and 20 fold, respectively, in endogenous ammonia. The diffusibility of ammonia may limit the use of a detoxifying gene, phosphinothricin acetyltransferase, as a selectable marker for alfalfa transformation. However, the addition of up to 40 times the standard levels of ammonium nitrate to the culture media used in this study had no effect on callus growth, although glutamine synthetase activity was inhibited by 50% and endogenous ammonia increased 27 fold. Therefore, ammonia accumulation may not be the primary cause of cell death in alfalfa after exposure to phosphinothricin. It follows that diffusion of ammonia from cell to cell would not restrict the selection for phosphinothricin acetyltransferase transformed cells, thereby indicating that this enzyme could be used as a selectable marker in transformation experiments.

Abbreviations: Phosphinothricin; PPT, Phosphinothricin acetyltransferase; PAT

Introduction

Based upon the ultimate goal of improving crop yield, there has been a major movement to genetically engineer plants resistant to herbicides that are both broad spectrum, and yet, biologically innocuous to non-target organisms (Botterman and Leemans 1988). In addition, there is a need for a wide range of selectable marker genes, beyond kanamycin resistance, which could be used in the transfer and selection of other agronomically useful traits.

Phosphinothricin (PPT, trade name Basta), a non-selective herbicide, is a phosphinic acid analogue of L-glutamic acid and inhibits glutamine synthetase (E.C. 6.3.1.2) in higher plants (Bayer et al. 1972; Leason et al. 1982; Ridley and McNally 1985; Manderscheid and Wild 1986). Primary incorporation, and recycling, of ammonia into organic compounds is generally accepted to occur via the glutamine synthetase-glutamate synthase pathway (Wild and Manderscheid 1984). Consequently, inhibition of glutamine synthetase results in a rapid accumulation of free ammonia, reported to be a primary factor in the herbicidal activity of PPT and decline in photosynthetic activity (Tachibana et al. 1986). However, several published reports conflict with respect to the relationships among ammonia accumulation, the loss of photosynthetic capability, and plant death (Wild and Manderscheid 1984; Sauer et al. 1987; Grumbles 1987; Wild et al. 1987).

While work towards establishing PPT's exact mode of action continues, De Block et al. (1987) have genetically transformed several plant species with a gene coding for phosphinothricin acetyltransferase (PAT) activity, which provides the plant with the ability to detoxify PPT. The bacteria *Streptomyces viridochromogenes* which synthesizes a tripeptide antibiotic containing PPT (Bayer et al. 1972), was the source of the bar gene coding for PAT, the detoxifying enzyme, PAT (Thompson et al. 1987). Using an *Agrobacterium* mediated transformation process this gene, placed under control of the 35S

Offprint requests to: M. A. Walker

cauliflower mosaic virus promoter, was successfully transferred to, and subsequently expressed in, tobacco, potato and tomato (De Block et al. 1987). Field testing of these plants has demonstrated that PPT can be used as a post-emergence herbicide in engineered plants (De Greef et al. 1988).

If ammonia accumulates to toxic levels after the inactivation of glutamine synthetase by PPT, and if this is the cause of cell death, then the use of PAT as a possible selectable marker may be limited. The limitation would occur because ammonia readily diffuses across cell membranes (Smith and Walker, 1978). In a mixed population of cells containing active and inactive forms of glutamine synthetase, it would therefore be expected that ammonia would accumulate in both cell types to almost equivalent concentrations. Therefore, in a plant transformation experiment using PPT as a selective agent, ammonia accumulating within nontransformed cells could diffuse into neighbouring transformed cells, thereby causing cell death. The observation that PPT can in fact be used successfully as a selective agent in transformation experiments using the bar gene as a selectable marker (De Block et al. 1987) argues against the accepted mode of action of this herbicide. The objectives in this study were therefore to compare the effects of PPT on alfalfa shoot tissue and cell cultures in terms of inactivation of glutamine synthetase and ammonia accumulation, and using cell cultures, to test whether the amount of ammonia accumulation found in PPT treated cell cultures can account for the toxicity of the herbicide.

Materials and methods

Plant Tissue and PPT Application. Alfalfa, *Medicago sativa* L., line RL34, was grown in a controlled environment room with day/night temperatures of 20° and 15°C, respectively, a 16 hr photoperiod, and a photon flux density of 250 umol $m^{-2}s^{-1}$. During experimental periods all plants were cut back prior to flowering and fertilized with a commercial fertilizer containing 20-20-20 NPK and micronutrients (Plant Products Co., Ajax, Ont). For PPT treatment, fresh healthy cuttings were excised 4-6 nodes below the apical tissue and the lower leaves removed, such that only the 3 upper, fully expanded leaves and the apex remained. Cuttings were immediately placed into individual tubes containing 10 ml of 1/8 strength of the above fertilizer solution. PPT (commercial grade Basta herbicide, 20% DL-PPT, Hoechst Chemical Co., Frankfurt), containing the surfactant Agral 90 (0.1% v/v), was applied as a foliar spray until all surfaces were wet. Treatment, and subsequent sampling periods, were always initiated 90 min after the beginning of the light period. Tissues were sampled at selected intervals after treatment as required.

Callus tissue was initiated from petiole explants which were surface sterilized with ethanol and 4% sodium hypochlorite. Three to five petioles were placed in a 9 cm petri dish containing 25 ml B_5h (Senaratna et al. 1990) medium and incubated at 25°C, with a 16 h photoperiod and photon flux density of 150 umol $m^{-2}s^{-1}$. After 2 weeks growth, calli were transferred to fresh B_5h medium containing the appropriate concentrations of ammonium or filter sterilized PPT and incubated as above, for 10 days. To initiate regrowth, petioles were transferred back to standard medium and incubated an additional 10 days.

Glutamine Synthetase Assay. The activity of glutamine synthetase was measured according to modified procedures of Rhodes et al. (1975) and Fraser and Ridley (1984). Tissue samples (0.05 g fresh weight of callus, or 15 19.5 mm^2 fresh leaf tissue discs) were placed into 1.5 ml microcentrifuge tubes, sealed, and frozen in liquid nitrogen for 15 s and subsequently thawed for 45 s at 32°C. This freeze-thaw process was repeated for a total of 5 cycles. Following the last freeze-thaw cycle, 200 ul of a reaction mixture containing 18 mM ATP, 45 mM $MgSO_4$, 6 mM hydroxylamine, 92 mM L-glutamate in imidazole-HCl buffer, pH 7.0 were added and the tubes incubated at 32°C for 15 min. After incubation, 100 ul of ferric chloride reagent (10 g TCA and 8 g ferric chloride in 250 ml 0.5 N HCl) were added to each sample and absorbance read at 498 nm on a Beckman DU-65 spectrophotometer supported by "Quant II Linear" software. Glutamyl hydroxymate was used as the standard.

Ammonia Analysis. Tissue samples (0.05 g fresh weight of callus, or 9 19.5 mm^2 fresh leaf discs) were homogenized, on ice, in 70 ul water and analyzed by a modification of the procedure of Novozamsky et al. (1974). After centrifugation, 10 ul of 4N TCA were added to each sample, the samples were then frozen, thawed, recentrifuged, and 50 ul aliquots of supernatant removed for analysis. To each aliquot 150 ul of ammonia reagent (see below) were added. After thorough mixing, 50 ul of phosphate buffer, pH 12, and 50 ul of 5% chlorine bleach were added. Samples were incubated at room temperature for 30 min, after which absorbance was read at 635 nm. Ammonia reagent was composed of 4N TCA, alkaline phenolate, and 4% (w/v) EDTA in a 5:2.5:0.025 (v/v/v) ratio. Alkaline phenolate contained 50 g NaOH and 24 ml 90% liquid phenol diluted to a final volume of 100 ml.

Results and Discussion

Foliar application of PPT, at concentrations of 10^{-3} M or higher, caused a significant reduction in leaf fresh weight, beginning at 12 h after treatment and reaching maximal injury at 48 h (Fig. 1). Concentrations of 10^{-5} M or lower were not effective. The loss in fresh weight is a symptom of herbicidal activity and cannot be overcome by the exogenous application of glutamine (Tachibana et al. 1986).

Increases in endogenous ammonia concentration in alfalfa shoot cuttings were noted, only 1 day after treatment with 10^{-3} M PPT or higher concentrations (Fig. 2). However, the level of ammonia accumulation was not dependent

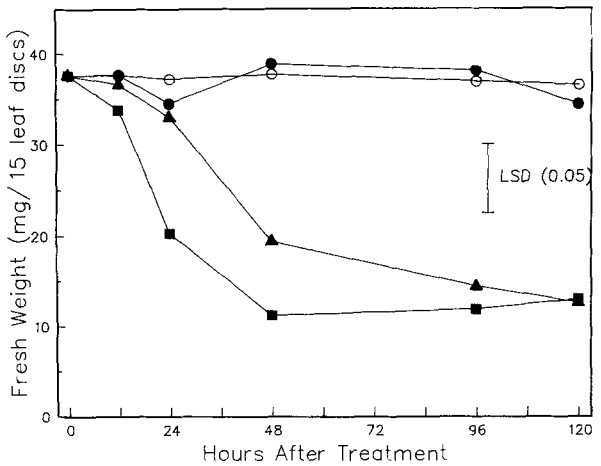

Fig. 1. Effect of foliar application of phosphinothricin (PPT) on leaf fresh weight of alfalfa (*Medicago sativa* L.). Plants were sprayed with 10^{-1} (■), 10^{-3} (▲) and 10^{-5} M (○) solutions of PPT and no PPT (●) as a control. The fresh weight represents the weight of 15 leaf disks of 19.5 mm^2 each taken at selected times after application of PPT. Least significant difference (LSD) between means is shown at p = 0.05, n = 3.

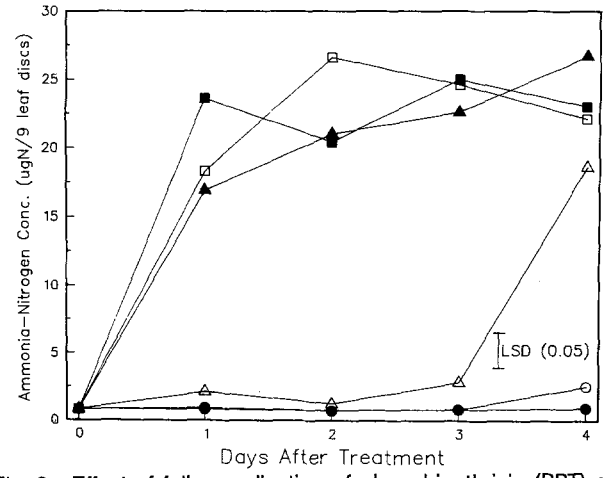

Fig. 2. Effect of foliar application of phosphinothricin (PPT) on ammonia concentration of leaves from alfalfa (*Medicago sativa* L.). Plants were sprayed with 10^{-1} (■), 10^{-2} (□), 10^{-3} (▲), 10^{-4} (△), and 10^{-5} M (○) solutions of PPT, and no PPT (●) as a control. The ammonia concentration of leaf discs was determined at selected times after application of PPT. Least significant difference (LSD) between means is shown at p = 0.05, n = 3.

on the concentration of PPT. A PPT application at 10^{-4} M also elevated ammonia concentration, but the increase was not evident until six days after the initial foliar application.

With a foliar application of 10^{-1} M PPT, glutamine synthetase activity was reduced at 12 h by 20% and inhibition increased slightly until 120 h (Table I). With 10^{-3} M PPT, there was a more obvious time dependent effect on glutamine

Table I. Effect of foliar applications of phosphinothricin (PPT) on glutamine synthetase activity in alfalfa (*Medicago sativa* L .) leaves.

PPT Conc. (M)	Glutamine Synthetase Activity[a] (% of control)				
	12 h	24 h	48 h	96 h	120 h
10^{-5}	88	100	100	100	100
10^{-3}	95	90	76	68	58
10^{-1}	80	81	72	80	75

[1]Glutamine synthetase activity of leaf discs was measured at selected times after application of PPT. The activity of control samples averaged 3.18 umol h^{-1} for 15 leaf discs, or 86 umol h^{-1} g^{-1} FW and were not significantly different at p = 0.05, n = 3 over time according to ANOVA.

synthetase inhibition with a gradual increase in the level of enzyme inhibition to over 40% at 120 h. PPT at a lower concentration (10^{-5} M), which did not cause a change in ammonia concentration (Fig. 1), did cause an initial transient level of inhibition, but this decrease in glutamine synthetase activity was not maintained over the course of the experiment (Table I). When PPT was incorporated directly into the enzyme reaction mixture, glutamine synthetase from leaves was inhibited by as much as 73% in a concentration dependent manner (Table II).

Table II. Effect of the addition of phosphinothricin (PPT) directly to the enzyme reaction mixture in the analysis of glutamine synthetase activity from alfalfa (*Medicago sativa* L.) leaves and callus.

PPT Conc. (M)	Alfalfa Leaf % of control	Alfalfa Petiole Callus % of control
10^{-6}	100	100
10^{-4}	100	100
10^{-3}	82	66
10^{-2}	73	50
10^{-1}	27	44

With no PPT added, glutamine synthetase activity for alfalfa leaf discs was 34 umol h^{-1} g^{-1} FW and for alfalfa petiole callus was 43 umol h^{-1} g^{-1} FW. Values are the mean of 2-3 determinations and std errors were less than 7%.

Alfalfa callus tissue appeared to be more sensitive than leaves to PPT as indicated by the reduced glutamine synthetase activity and ammonia accumulation at PPT concentrations as low as 10^{-6} M (Table III). After transfer of the PPT-treated callus to PPT-free media, no regrowth

was observed for 10^{-4} and 10^{-2} M treated tissue, whereas 10^{-6} M treatments did permit callus regrowth (Table III). When PPT was incorporated directly into the enzyme reaction mixture, glutamine synthetase from the callus was also inhibited in a concentration dependent manner (Table II). Enhanced sensitivity to lower concentrations of PPT has also been reported for assays dealing with purified enzyme (Leason et al. 1982; Mandersheid and Wild; 1986) or isolated cells (Fraser and Ridley 1984). Thus, PPT in the range of 10^{-3} M does elicit symptoms, in both leaf and callus tissue, similar to those reported for other plant species, specifically, decreased in glutamine synthetase activity and accumulation of ammonia (Leason et al. 1982; Fraser and Ridley 1984; Wild et al. 1987). These data support the findings of Tachibana et al. (1986) who suggested that ammonia accumulation was the primary factor in PPT toxicity.

Table III. Effect of phosphinothricin (PPT) in the culture medium on glutamine synthetase activity, ammonium concentration and regrowth of alfalfa (*Medicago sativa* L.) petiole callus tissue. Callus regrowth was scored as the percentage of petioles exhibiting callus formation after transfer to fresh media without PPT.

PPT Conc (M)	Ammonium in callus (ug NH_4 g^{-1} FW)	Glutamine Synthetase (umol h^{-1} g^{-1} FW)	Callus Regrowth (%)
0	44 ± 16	55 ± 4.8	100
10^{-6}	86 ± 26	34 ± 2.4	83
10^{-4}	206 ± 12	33 ± 6.4	0
10^{-2}	462 ± 88	27 ± 9.6	0

Values represent the mean of 3 determinations ± Std. Error. Callus was held on phosphinothricin media for 14 days.

To test this hypothesis further, callus was grown on culture media containing up to 40 times the standard levels of ammonium nitrate. Glutamine synthetase activity was reduced by up to 50% and endogenous ammonia levels increased to approximately 27 times that of control; however, there was no effect on callus regrowth after transfer to standard media (Table IV). Ammonia concentrations attained in the callus exposed to the excess ammonium nitrate exceeded those levels found after treatment with 10^{-2} M PPT (compare Tables III and IV), and yet had no effect on the regrowth potential of the callus. Additionally, callus from the excess ammonia concentration treatments remained highly embryogenic whereas embryogenesis was completely blocked with PPT treatment .

Therefore, ammonia accumulation per se may not be the cause of cell death in PPT-treated alfalfa petiole callus. It follows that diffusion of ammonia, endogenously produced by susceptible plant cells exposed to PPT, would not restrict the selection for PAT transformed cells. Thus, there is no reason to suspect that PAT could not be successfully used as a selectable marker in plant transformation experiments.

Table IV. Effect of ammonium nitrate in the culture medium on glutamine synthetase activity, ammonium concentration and regrowth of alfalfa (*Medicago sativa* L.) petiole callus tissue. Callus regrowth was scored as the percentage of petioles exhibiting callus formation after transfer to fresh media without PPT.

Added Ammonium (mM)	Ammonium in callus (ug NH_4 g^{-1} FW)	Glutamine Synthetase (umol h^{-1} g^{-1} FW)	Callus Regrowth (%)
10	44 ± 16	55 ± 4.8	100
40	72 ± 6	35 ± 4.8	100
100	154 ± 34	38 ± 4.0	83
200	356 ± 28	34 ± 2.4	100
400	1092 ± 72	28 ± 0.1	100

Values represent the mean of 3 determinations ± Std. Error. Callus was held on phosphinothricin media for 14 days.

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