SECTION 4

HERBICIDE BIOCHEMISTRY, HERBICIDE METABOLISM AND THE RESIDUES IN GLUFOSINATE-AMMONIUM (PHOSPHINOTHRICIN) – TOLERANT TRANSGENIC PLANTS

Summary Note

This document summarises the information available on the herbicide biochemistry, the herbicide metabolism and the residues in glufosinate-ammonium (phosphinothricin)-tolerant transgenic plants.

Scope of this document: This document is limited to a condensed discussion of the herbicide biochemistry and metabolism specifically in glufosinate-ammonium (phosphinothricin)-tolerant transgenic plants. It is not intended to be an encyclopaedic review of all scientific experimentation with glufosinate tolerant plants or with the herbicide glufosinate itself. Especially, this document is not to be confused with the type of dossier currently composed for plant pesticides according to directive 91/414/EEC. Moreover, it does not discuss the plentiful information available on the use of the herbicide in agricultural and other applications. Food safety aspects of the use of glufosinate-ammonium on glufosinate-ammonium-tolerant plants are beyond the scope of this document. Such information is available from other sources, including the respective governmental organisations regulating herbicide use.

1. Biochemistry and Physiology of the Herbicide in Non-tolerant and in genetically Modified Glufosinate (Phosphinothricin) – Tolerant Plants

Glufosinate (phosphinothricin; DL-homoalanin-4-yl(methyl)phosphinic acid) is a racemic phosphinico amino acid (Hoerlein, 1994). Its ammonium salt (glufosinate-ammonium) is widely used as a non-selective herbicide and is the active ingredient of the commercial herbicide formulations Basta®, Buster®, Challenge®, Conquest®, Dash®, Final®, Finale®, Liberty® and Ignite®. The L-isomer of glufosinate is a structural analogue of glutamate and, therefore, is a competitive inhibitor of the enzyme glutamine synthetase (GS) of bacteria and plants (Bayer *et al*., 1972; Leason *et al*., 1982). The D-isomer is not a GS inhibitor and is not herbicidally active.

Due to the inhibition of GS, non-tolerant plant cells accumulate large amounts of toxic ammonia produced by nitrate assimilation and photorespiration (Tachibana *et al*., 1986) and the level of available glutamine drops (Sauer *et al*., 1987). Damage of cell membranes and inhibition of photosynthesis are followed by plant cell death. The action of glufosinate is dependent on environmental conditions. Temperatures below 10°C, as well as drought stress, reduce its efficacy because of the limited metabolic activity of the plant (Donn, 1982). Also, light is an important factor for the action of glufosinate (Koecher, 1983).

In genetically modified glufosinate-tolerant plants, the L-isomer of glufosinate is rapidly metabolized by the action of the enzyme phosphinothricin acetyltransferase (PAT) into the non-phytotoxic stable metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic

acid). N-acetyl-L-glufosinate does not inhibit glutamine synthetase. Therefore, no phytotoxic physiological effects are observed in genetically modified glufosinate-tolerant plants.

Glufosinate is a contact herbicide and is taken up by the plant primarily through the leaves (Haas and Müller, 1986). There is no uptake from the soil through the roots, presumably because of the rapid degradation of glufosinate by soil micro-organisms. There is limited translocation of glufosinate within the plant. After application of L-glufosinate, N-acetyl-L-glufosinate and further metabolites on distinct leaves, a preferential transport into the upper leaves and a low level of translocation into the lower plant parts was observed in both genetically modified and unmodified tobacco plants (Droege, 1991; Droege-Laser *et al*., 1994).

Glufosinate has a wide spectrum of activity encompassing monocotyledonous and dicotyledonous species. Due to its limited systemic action, there is no enduring effect on perennial weeds. Examples of weed species that are not, or only weakly, combated by glufosinate are *Viola arvensis, Bromus spp., Lolium spp., Agropyron repens* and *Urtica urens* (Hoechst, 1991). Weeds emerging after herbicide application are not affected.

Glufosinate is rapidly broken down in soil due to microbial degradation. At 20°C, the soil half-life is less than 10 days (Smith, 1988; Dorn *et al*., 1992). Metabolites arise from oxidative deamination and from acetylation (Dorn *et al*., 1992). L-glufosinate can be used by micro-organisms as a source of nitrogen (Tebbe and Reber, 1989). There are no special reports on the degradation of the D-enantiomer in soil, however, the fast dissipation of the DL-racemic mixture was found in all soils investigated under laboratory, as well as, field conditions (Dorn *et al*., 1992; Smith, 1989). The end products of microbial degradation are CO_2 and natural phosphorus compounds. There is also formation of bound residues which are finally mineralized (Dorn *et al*., 1992).

2. Metabolism of Glufosinate-ammonium in Genetically Modified Plants in Comparison to Non-Transgenic Plants

Because of the widespread use of glufosinate in agricultural practices (non-selective application, as a desiccant, selective application in tolerant crops), the metabolism of glufosinate in sensitive, as well as in glufosinate-tolerant plants, is addressed. If the PAT enzyme is used as part of selectable marker systems of genetically modified plants, lower levels of PAT activity are required compared to glufosinate-tolerant crops for selective field applications of the herbicide.

The metabolism of glufosinate in artificial systems like cell suspension cultures (soybean, wheat, maize) and sterile plants (tobacco, alfalfa, carrot) has been analyzed by Komossa and Sandermann (1992) and by Droege-Laser *et al*. (1994). After treatment of non-transgenic plants with glufosinate, the unstable intermediate 4-methylphosphinico-2-oxo-butanoic acid (PPO) is formed via deamination. A rapid decarboxylation reaction then results in the stable main metabolite 3-methylphosphinico-propionic acid (MPP) which is non-phytotoxic. Within non-transgenic plants, PPO can also be reduced to form 4-methyl-phosphinico-2-hydroxy-butanoic acid, another final and stable product (Droege-Laser *et al*., 1994). In contrast to transgenic PAT-expressing plants, there is no direct proof that in non-tolerant plants only the L-isomer is metabolized.

The metabolism of glufosinate in non-tolerant plants is only limited because plants rapidly die after herbicide application. Moreover, if used as a non-selective herbicide in agricultural practice, glufosinate is not intended to be applied directly, except for desiccation purposes. If crop plants have not emerged at the time of application, residues in the crop plants can only be due to uptake from the soil. Studies evaluating the amount and nature of “indirect” uptake have shown that traces, mainly of the major metabolite 3-methylphosphinico-propionic acid (MPP), can be found (Hoerlein, 1994). This

non-phytotoxic metabolite is also a well known soil metabolite (Tebbe and Reber, 1988) which can be taken up by the roots. It was found to be the only relevant residue following normal weed control in non-transgenic plants (Hoerlein, 1994). In desiccation, residues consist of unchanged glufosinate, with small portions of MPP and a non-relevant portion of 2-methyl-phosphinico-acetic acid.

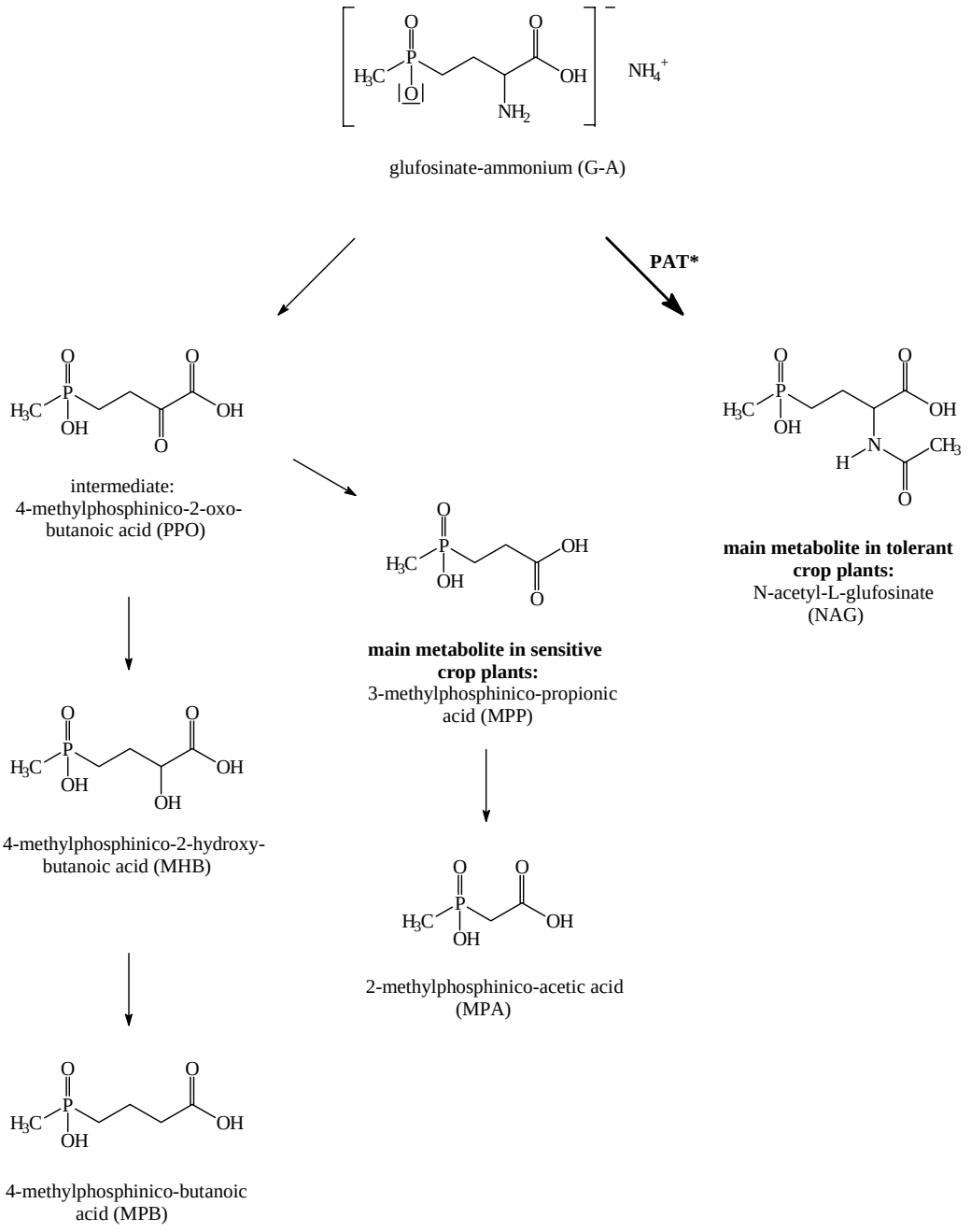
The insertion of genes encoding phosphinothricin acetyltransferase (PAT) enables plants genetically modified in this way to rapidly metabolize the herbicidal active moiety of glufosinate-ammonium into the non-phytotoxic metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid). This metabolite is not found in non-transgenic plants.

The metabolism of glufosinate-ammonium following direct application on genetically modified glufosinate-tolerant corn (maize), oilseed rape (canola), tomato, soybean and sugar beet (*Figure 2*.3) has been investigated with the formulated test substance (Burnett, 1994; Tshabalala, 1993; Thalacker, 1994; Stumpf, 1995; Rupprecht and Smith, 1994; Rupprecht *et al*., 1995; Allan, 1996). In all glufosinate-tolerant crops, the principal residues were N-acetyl-L-glufosinate and - usually with lower concentrations – glufosinate-ammonium and MPP. In corn grain and rape seed, the main residue identified was MPP, with lower concentrations of N-acetyl-L-glufosinate. In corn forage, in soybean seed, in sugar beet roots and in tomato fruit, the main residue was N-acetyl-L-glufosinate. Experiments of Droege *et al*. (1992) and Droege-Laser *et al*. (1994) using transgenic tobacco, carrot, and alfalfa plants also found N-acetyl-L-glufosinate as the major metabolite in glufosinate-tolerant plants. Besides the principal residues, trace levels of other metabolites were also identified in soybean including 2-methylphosphinico-acetic acid (MPA) and 4-methylphosphinico-butanoic acid (MPB). The herbicidally inactive D-glufosinate appears to be stable in plants due to the L-specific acetylation activity of the PAT enzyme (Droege *et al*., 1992).

In genetically modified glufosinate-tolerant plants expressing the PAT enzyme, it appears that two metabolic routes compete: (1) the deamination of glufosinate and subsequent conversion of 4-methyl-phosphinico-2-oxo-butanoic acid (PPO) to 3-methylphosphinico-propionic acid (MPP) or to 4-methyl-phosphinico-2-hydroxy-butanoic acid, and (2) the N-acetylation of L-glufosinate by PAT (Droege-Laser *et al*., 1994). The second of these two routes predominates when PAT specific activity is relatively high.

If genetically modified plants express the PAT enzyme at a low level, the deamination pathway with the formation of MPP predominates. In this case, besides substantial amounts of the acetylated and non-acetylated forms of L-glufosinate, the metabolites 4-methyl-phosphinico-2-oxo-butanoic acid (PPO), 3-methylphosphinico-propionic acid (MPP) and 4-methyl-phosphinico-2-hydroxy-butanoic acid are formed (Droege-Laser *et al*., 1994).

Figure 2.3 **Metabolism of Glufosinate-Ammonium in Non-Transgenic and in Transgenic, Tolerant Crop Plants (Corn, Oilseed rape, Tomato, Soybean, Sugar beet)**



*) PAT = phosphinothricin acetyl - transferase

Source : derived from FAO, 1998

3. Metabolites and Residues in Genetically modified Plants

The FAO's Joint Meeting of Experts on Pesticide Residues (JMPR) suggested, in 1998, a revised residue definition, considering the nature of the residue occurring in conventional and transgenic glufosinate-tolerant plants. This definition was confirmed by the 1999 JMPR as suitable for the establishment of maximum residue levels and for the estimation of dietary intake. For glufosinate-ammonium, residue is defined as the sum of glufosinate-ammonium, MPP and N-acetyl-L-glufosinate (FAO, 1998).

For residue studies, glufosinate-ammonium and the principal metabolites N-acetyl-glufosinate and 3-methylphosphinico-propionic acid (MPP) are extracted from finely ground sample material with water. After cleaning-up of the extracts, the residues are derivatised, resulting in the formation of methylated/acetylated derivatives. These are cleaned up and determined by gas chromatography using a phosphorus-specific flame photometric detector, yielding analytical recoveries which are satisfactory on many substrates. Glufosinate-ammonium and N-acetyl-L-glufosinate are determined as a common derivative and MPP is quantified as a separate derivative. If a differentiation between glufosinate-ammonium and N-acetyl-L-glufosinate is required, the two compounds need to be separated prior to derivatisation.

Using this procedure, the following individual total residues represented as the sum of glufosinate-ammonium, N-acetyl-L-glufosinate and MPP were obtained from genetically modified, glufosinate-tolerant plants while the limit of quantification for each analyte was 0.05 mg/kg. Individual residue data are mainly part of national submissions for glufosinate-ammonium.

A. Oilseed rape

At an application rate of 750 g/ha or 2 x 800 g/ha, the total residue in the seed at harvest encompasses between < 0.05 and 0.24 mg/kg. Rapeseed oil was found to contain below 0.05 mg/kg total residue.

B. Corn

At an application rate of 400 + 500 g/ha or 2 x 800 g/ha, the total residue in corn grain was between < 0.05 and 0.07 mg/kg. Corn oil contained less than 0.05 mg/kg total residue.

C. Soybean

At an application rate of 400 + 500 g/ha, the total residue in soybean seed ranged from 0.32 to 1.88 mg/kg.

D. Sugar beet

At an application rate of 2 x 600 g/ha or 2 x 800 g/ha, the total residue in roots which are relevant to human nutrition as a raw material for sugar production, were found to be between < 0.05 and 0.88 mg/kg. Refined sugar after processing contained no residues (< 0.05 mg/kg).

The lowest NOEL (no observed effect level), established in a chronic (24 months) feeding study in rats, was 2 mg glufosinate-ammonium/kg body weight/day (Ebert *et al*., 1990). This low toxicity is due to the mode of action of glufosinate. In mammals, glufosinate-ammonium competitively inhibits glutamine synthetase (GS). However, contrary to the situation in plants, fixation of ammonia is guaranteed by several metabolic pathways in order to maintain homeostasis of the amino acid pool.

The biosynthesis of glutamine from glutamate forms only one of the possibilities for fixation of ammonia and amino groups. Thus GS is only of minor importance for ammonia fixation in mammals. In this context, Hack *et al*. (1994) found that inhibition of glutamine synthetase by glufosinate did not essentially affect the level of ammonia, glutamate and other amino acids. Since the toxicological data indicated no genotoxic, carcinogenic or teratogenic potential, an acceptable daily intake (ADI) value of 0.02 mg/kg body weight/day was accepted for glufosinate (WHO, 1992). This value has been confirmed as group ADI for glufosinate-ammonium, MPP and N-acetyl-L-glufosinate (WHO, 1999).

Tolerances for combined residues of glufosinate-ammonium and its metabolites (3-methylphosphinicopropionic acid and N-acetyl-L-glufosinate) have been established in the USA for transgenic field corn and transgenic soybean. The tolerances are 0.2 mg/kg and 2.0 mg/kg for corn grain and for soybean seed, respectively (EPA, 1999).

Glufosinate-ammonium is registered for the use in the following transgenic tolerant crops:

Canada	Canola and Corn
USA	Corn and Soybean
Germany	Corn
Portugal	Corn
Argentina	Corn
Romania	Corn

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