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EDITORIAL

On glyphosate

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Abstract **–** This Editorial briefly discusses the current issues surrounding glyphosate - the most controversial pesticide active ingredient of our time. The paper pays special attention to the effects of glyphosate on plant-pathogen interactions.

Keywords – glyphosate, plant-pathogen interactions, environment, human health, ecocycles, sustainability

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- In nature nothing exists alone.
Rachel Carson in "Silent Spring" (Carson, 1962)

- Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, daß ein Ding kein Gift sei. (All things are poison, and nothing is without poison: the dose alone makes a thing not poison).
Paracelsus in "Sieben Verteidigungsreden" (von Hohenheim, 1538)

Brief history

Glyphosate (N-[phosphonomethyl]glycine), a widely applied non-selective systemic herbicide, is bit by bit becoming the most controversial pesticide product ever produced. The molecule has a very simple, still rather unique zwitterionic chemical structure (Figure 1): unlike most other pesticides it is highly soluble in water and, depending on the soil properties, it may be very strongly bound to soil matrices.

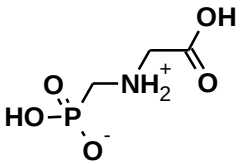

Figure 1. Chemical structure of glyphosate.

The chemical (that was decades later named as glyphosate) was first synthesized in 1950 at the Swiss company CILAG (Franz et al., 1997). About ten years later the herbicide manufacturer Stauffer Chemical Company patented it as a chelator (complexing agent for metals such as magnesium, copper, and zinc) (Fon and Uhing, 1964). To their later deep regret, researchers of the company missed to identify the molecule as a potential herbicide because of the short duration of the company's standardized biological assays (only five days, while the first, glyphosate-induced phytotoxic symptoms usually appear after about one week) (F. M. Pallos, personal communication in 1982). It took another ten years until the molecule's herbicidal properties were discovered at Monsanto Chemical Company* (Irani, 1969). The chemical was patented, obtained "glyphosate" as the common name, and the production of the first glyphosate-based commercial herbicide ("RoundUp") began in 1974 (Szekacs and Darvas 2012). In 2002 glyphosate was additionally patented by Monsanto as an antibiotic (Abraham, 2010).

At first, as a non-selective, so-called total herbicide, glyphosate had a narrow market, although its unique properties (low price, low mammalian toxicity, high efficacy due to a unique systemic action *via* basipetal and acropetal translocation) were rapidly acknowledged. The fact that glyphosate may lose its phytotoxic activity when contacted with the soil was first attributed to rapid degradation of the active ingredient to non-phytotoxic metabolites, but later explained by the binding of glyphosate molecules to soil particles (Duke et al., 2012). For many years, glyphosate has been considered as a "virtually ideal" herbicide (Duke and Powles, 2008). *Nota bene*, as it turned out later, the mechanism of binding of glyphosate to soil particles is very similar to that of

* Note added in proof: on September 14, 2016 Monsanto Chemical Company was acquried by Bayer AG.

phosphate. Thus, as a result of a displacement reaction, the leaching of soil-bound glyphosate to the groundwater is increased by phosphate fertilizers (Munira et al., 2016).

Still, the real breakthrough for glyphosate came only with the advent of the genetically modified (GM) crops: a field in which Monsanto pioneered (Komives, 2016). Glyphosate resistance of transgenic crops was achieved by introducing a second, non-sensitive target enzyme (from *Agrobacterium*, see later) in addition to the existing sensitive ones in selected crops (Pollegioni et al., 2011). The bacterial gene in the GM crops transformed them to be resistant ("RoundUp Ready") to the herbicide that otherwise kills all plants in the field, including the non-GM crops, too. However, the use of an insensitive EPSP implies that glyphosate itself remains unmetabolized in the tissues of resistant plants – in contrast to glufosinate-tolerant GM plants, in which the resistance is due to their ability to chemically modify (by an introduced bacterial enzyme via N-acetylation) the active ingredient thereby making it much less toxic to plants and to the environment (Muller et al., 2001).

Glyphosate-resistant GM crops have been in production for twenty years now, and their planting area is still increasing – making glyphosate the most-used pesticide in the world with a predicted annual sale of 1.35 million metric tons in 2017 (Newman et al., 2016).

Biological mode and mechanism of herbicidal action

Jaworski (1972) suggested that the phytotoxic action of glyphosate is due to an interference with the biosynthesis of L-phenylalanine (Figure 2) and, in particular, with the metabolism of chorismic acid (Figure 2), a key metabolite in the biosynthesis of the so-called aromatic amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan; Figure 2). An inhibition of the formation of aromatic amino acids certainly leads to toxic symptoms, since it leads to a halt of protein *de-novo* synthesis and production of the main plant growth hormone indole-3-acetic acid (a derivative of L-tryptophan).

An alternative mode of action was suggested by Duke and Hoagland (1978). They found elevated levels of phenolic secondary metabolites and also of the key enzyme of phenolic biosynthesis (phenylalanine ammonia-lyase, PAL, EC 4.3.1.5) in the roots of dark-grown, glyphosate-treated maize seedlings, and concluded that the phytotoxic action of glyphosate might be due to a stimulation of the biosynthesis of growth-inhibiting phenolics (Duke and Hoagland, 1978).

The exact biochemical mechanism of action of glyphosate was identified by Steinrücken and Amrhein (1980) as the specific inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19; Figure 3) confirming the findings of Jaworski (1972).

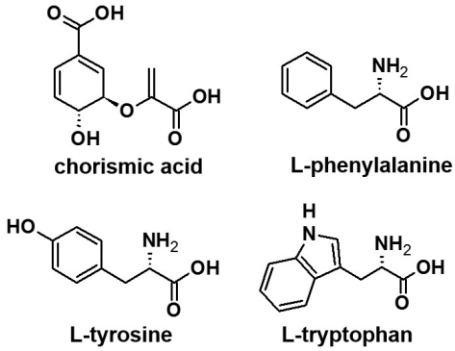

Figure 2. Chemical structures of chorismic acid and the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan.

The subsequent lack of aromatic amino acids in glyphosate-treated plants with time (but not immediately as observed by Duke and Hoagland, 1978) results in pleiotropic effects on the physiology of plants: reduced photosynthesis, growth retardation, oxidative stress, etc. (Gomes et al., 2014). It was also suggested that part of the phytotoxic effects of glyphosate may be due to the accumulation of shikimic acid and shikimic acid-3-phosphate (Figure 3) as a result of EPSP synthase inhibition (de Maria et al., 2006).

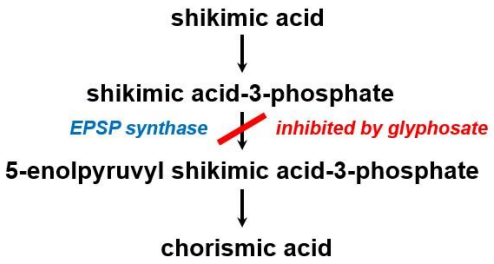

Figure 3. Phytotoxic mechanism of action of glyphosate: inhibition of the enzyme EPSP synthase

Effects on the secondary metabolism of plants

Aromatic amino acids are starting materials of a large number of chemicals plants synthesize. These chemicals are called secondary metabolites and they have important functions in plant life affecting responses to biotic and abiotic environmental stress, interactions with other (beneficial or harmful) organisms, and normal growth and development (Vogt, 2010). But deriving from chorismate, a key metabolite originated from the shikimic acid cycle (Fig. 4), multiple other metabolic pathways can also be influenced by glyphosate.

Early mode of action studies (Duke and Hoagland, 1978) suggested an induction of the phenolic secondary

product biosynthesis. In contrast, later investigations confirmed that synthesis of phenolic compounds originated from phenylalanine were stopped by glyphosate in plants (Holliday and Keen, 1982; Canal et al., 1987; Carbonari et al., 2014). Under scrutiny, it was also demonstrated that levels of some phenolic compounds (hydroxybenzoic acids) not directly synthesized by the shikimic acid pathway were induced by glyphosate in several plant species. The authors concluded that hydroxybenzoic acids could be overflow metabolites of the shikimic acid pathway. (Lydon and Duke, 1988).

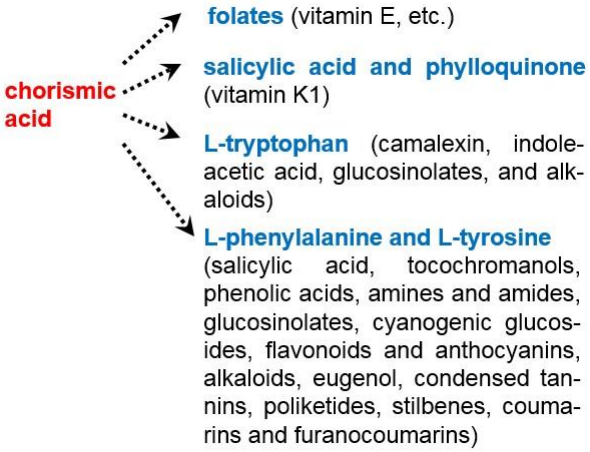

Figure 4. Synthetic routes of the major classes of plant metabolites derived from chorismic acid

A recent study reported on the responses of adventitious root cultures of *Echinacea purpurea* to glyphosate-induced starvation. Glyphosate reduced tissue growth and the levels of phenolic secondary metabolites. Feeding of L-tryptophan and, to a lesser extent, L-phenylalanine counteracted the phytotoxic effects of the herbicide and led to the accumulation of higher levels of secondary metabolites (Mobin et al., 2015). The authors concluded that in *Echinacea purpurea* plants the phytotoxicity of glyphosate affects the indolic pathway significantly stronger than the phenolic pathway.

The main building blocks of lignins are produced from L-phenylalanine via the phenolic biosynthesis pathway. Lignins are cross-linked, structural polymers in plant tissues. They are important components of cell walls: protect the cells against physical injury and also against pathogen attack. Glyphosate strongly inhibits lignification, making the plants physically weaker and also prone to infection by pathogens (Zobiole et al., 2010).

Further studies indicated glyphosate-induced disturbances in the biosynthesis of glucosinolate phytoalexins (Pedras and Yaya, 2014; Petersen et al., 2007), flavonoids (Margna et al., 1988), cocaine (Casale and Lydon, 2007), and other secondary metabolites from aromatic amino acids (Tzin and Galili, 2010).

Fate in the environment and side effects

Sprayed on the leaves glyphosate is rapidly taken up and translocated into the newly developing plant parts (Klier et al., 2008), and appreciable amounts are exuded from the roots into the soil solution (Coupland and Caseley, 1979). In many soils, glyphosate is not readily decomposed and may remain there for a long period of time unchanged or chelated with soil cations.

Not surprisingly, the continuous use of very large amounts of glyphosate led to environmental problems: first, glyphosate-resistant weeds started to appear in the fields (Nandula 2010). At the same time residues of glyphosate and its primary metabolite aminomethyl-phosphonic acid (AMPA, Figure 5) were detected in the soil, in freshwater, and in the organisms that live there (Perez et al., 2011), in the crop products, in the animals that were fed with them, and in humans, too. Therefore, studies were initiated to determine whether continuous exposure to glyphosate leads to unwanted effects to the environment, such as soil microorganisms (Kremer and Means, 2009), insects (Saska et al., 2016), fish (Giannini, 2013), and to humans. Results of these studies were presented in a number of papers (Szekacs and Darvas, 2012; Myers et al., 2016; Mensah et al., 2015) and discussed in excellent books (to mention one: Nandula, 2010).

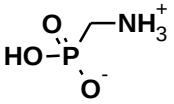

Figure 5. Chemical structure of aminomethylphospho-nic acid (AMPA). Breakdown of glyphosate to this metabolite has so far only been proven in soil microbes.

Most pesticide products contain substances in addition to the active ingredient(s) that are referred to as "inerts", adjuvants or co-formulants, e.g. increasing the penetration of the active. *Nota bene*, the science of toxicology does not know about "inert" chemicals: this would be against the law of Paracelsus (von Hohenheim, 1538). Consequently, those adjuvants and their glyphosate formulations have gone under scrutiny (Mesnage et al. 2013a), also in conjunction with parallel exposure to *Bacillus thuringiensis* toxins (Mesnage et al. 2013b). As regards to glyphosate-based herbicides, it is still not clear how many or what percentage of its side effects described in the scientific literature are due to those "inert" components components (Szekacs et al., 2014; Defarge et al., 2016). However, a certain group of adjuvants, the frequently used tallow amines, have been banned recently (AgriLand 2016).

Unfortunately, the results of studies on the side effects of glyphosate led to a number of conflicting

conclusions. For example, nephropathy, cancer, etc. was attributed to glyphosate. Originally, these findings appeared in the journal Food and Chemical Toxicology in September 2012, but the paper was retracted by the journal's editor in November 2013. The paper republished a year later (Seralini et al., 2014). Recently, glyphosate was shown to induce birth defects in rats (Guerrero Schimpf et al., 2016) and was suggested (without experimental evidence) to produce DNA and epigenetic effects (Nardemir et al., 2015), and to act as the analogue of the amino acid glycine to replace it in diverse proteins (Samsel and Seneff, 2016). In contrast, glyphosate and its AMPA metabolite were found to inhibit the proliferation and promote the apoptosis of cancer cells (but not normal ones), leading to a suggestion that they may be lead molecules to new anticancer therapies (Li et al., 2013).

Effects on plant-pathogen interactions

Another intriguing subject is the very complex influence glyphosate may exert on plant-pathogen interactions. First, glyphosate is antimicrobial *per se* (Morjan et al., 2002; Samac and Foster-Hartnett, 2012). Second: glyphosate may alter the chemical, physical, and biochemical mechanisms plants use to fend off to pathogens. Some of the protection barriers are constitutive, such as the cell walls and waxy epidermal cuticles of the leaves. Furthermore, plant cells are capable of detecting attacking pathogens and react with inducible responses, *e.g.*, the production of toxic chemicals (hydrogen peroxide and other reactive oxygen species, phytoalexins, *etc.*), pathogen-degrading enzymes, and deliberate cell suicide (hypersensitive reaction, HR) (Barna et al. 2012). Saska et al. (2016) found that population parameters of the aphid *Metopolophium dirhodum* were significantly negatively affected by applications of sub-lethal doses of glyphosate. Glyphosate, by blocking the synthesis of chorismic acid (and the aromatic amino acids) may negatively influence virtually all of the induced defense plant responses, and, after longer times of exposure, even the constitutive ones. To mention a few:

1) Higher levels of the natural antimicrobial products (called phytoalexins) are synthesized at infection sites (Mhlongo et al., 2016). 2) New lignin (built from hydroxylated benzoic acids) in cell walls and localized at the point of infection keeps the pathogen isolated and blocks its spread in the tissues. 3) Salicylic acid (a signal compound in plant response to pathogen infection) is produced by two parallel routes, both of which need intermediates (chorismic acid and L-phenylalanine) from the shikimic acid pathway. It is interesting to note that salicylic acid antagonizes the biological activity of glyphosate (Akbulut, 2014). 4) Naturally, the synthesis a wide variety of pathogenesis-related proteins that are important for plant resistance depends on the availability of their aromatic amino acid building blocks (Johal and Huber, 2009).

Since most phytoalexins are synthesized from L-phenylalanine, their levels are strongly reduced in glyphosate-treated plants (Keen et al., 1982) leading to a synergistic toxic action between the herbicide and the pathogen (Levesque et al., 1993; Uotila et al., 1994; Descalzo et al., 1996). In fact, herbicidal activity of glyphosate on bean seedlings was partly attributed to parasitization of plants by soilborne fungi (Johal and Rahe, 1984; Johal and Rahe, 1988; Johal and Huber, 2009). This suggestion was confirmed in later investigations by Schafer et al. (2012 and 2013), who concluded that rhizosphere interactions play a major role in the phytotoxic action of glyphosate and might be involved in the evolution of weed tolerance and resistance to glyphosate in the field. A practical application of the phenomenon of synergistic phytotoxic action between pathogens and glyphosate was invented by Sharon et al. (1992): they increased the weed controlling ability of the *Alternaria crassicae*-based mycoherbicide by adding very low (non-phytotoxic) concentrations of glyphosate. Unexpectedly, glyphosate did not influence the pathogenicity of *Fusarium solani* f. sp. *pisi* to pea seedlings (Kawate et al., 1992), although pisatin (the key phytoalexin of this plant) is also a product of the phenolic biosynthesis pathway (Komives and Casida, 1983).

Hypersensitive response was considered for a long time a key process of plant disease resistance until Kiraly et al. (1972) showed that HR is a consequence, and not the cause of plant resistance to infections. Interestingly, glyphosate had no effect on the hypersensitive responses of the host cells in leaves infected with incompatible bacteria (Holliday and Keen, 1982). Bacterial counts showed that inhibition of the biosynthesis of the phytoalexin glyceollin by glyphosate only partially prevented the expression of resistance to bacteria. In contrast, glyphosate-treated plants, fed with phenylalanine and tyrosine, were capable to synthesize and accumulate glyceollin and were also resistant to incompatible bacteria. These results were interpreted on the basis that glyceollin accumulation is only a component of the complex mechanism of resistance to bacteria (Holliday and Keen, 1982).

In addition to their roles as phytoalexins, phenolic stress metabolites may contribute to plant resistance to pathogens via taking part in complex redox reactions that lead to the formation of antimicrobial reactive oxygen species and quinines (Boeckx et al., 2015). Since glyphosate inhibits only the formation of phenols originated from intermediates after chorismic acid, phenols derived from earlier steps (such as hydroxybenzoic acids) may be important for the production of reactive oxygen species.

Salicylic acid plays a central role in plant defense signaling, regulating multiple biochemical pathways

(Wang 1014). Since glyphosate blocks both synthetic routes leading to salicylic acid, some of its effects on plant pathogen interactions may be due to the absence of this key molecule. Unfortunately, no study was published yet on the salicylic acid levels in glyphosate-treated plants.

Future prospects

Although there are significant arguments why glyphosate should be banned, most probably it will remain in use in the foreseeable future. However, under such conditions, it is clear that further, thorough and rather elaborate investigations are necessary to unequivocally determine the environmental and human health risks of use of glyphosate, its by-products and formulations. In this respect "omics" studies, such as those of Martyniuk and Simmons (2016) and Duke et al. (2013), could be the most promising. Additional research is also needed on the biological functions and the regulation of the individual steps in the biosynthesis of secondary plant metabolites in order to elucidate the antagonistic (and possibly synergistic) interactions of different metabolites derived from the shikimic acid pathway.

A further but not less important challenge for future research is the identification of key mechanisms by which residues of glyphosate may affect ecosystems and ecological cycles.

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