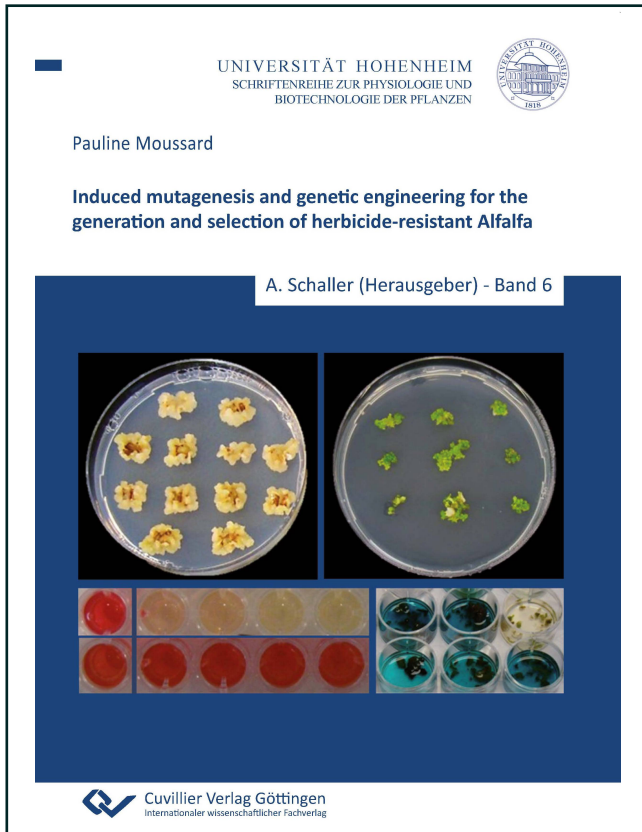




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Induced mutagenesis and genetic engineering for the generation and selection of herbicide-resistant Alfalfa



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1 INTRODUCTION AND OBJECTIVES

1.1 CHEMICAL WEED CONTROL AND HERBICIDE-RESISTANCE

Weeds are plants growing where they are not wanted and where they have not been intentionally sown and thus competing with the desirable crops for space, light, water and nutrients. As weeds are naturally strong and persistent competitors because of their abundant seed production, their physical adaptation for seed spreading and their vegetative reproductive structures dedicated to rapid and efficient population establishment, they always tend to dominate the crops and invade the fields. Thus, various problems arise in crop fields as a result of weed infestation: decrease of crop purity and quality, presence of allergens or toxins to humans or livestock, presence of a host for crop disease and high impact on the farm economy due to crop losses.

Therefore, farmers developed weed management techniques in order to efficiently fight weed infestation. Common methods used to control weeds in a crop field include preventive measures to avoid the introduction of weeds such as cleaning the whole equipment, planting weed-free certified crop seeds, but also cultural practices to maintain a healthy crop better competing with weeds and preventing their establishment, mechanical practices to destroy weeds (mowing, plowing, hand-pulling, digging, mulching...), as well as biological weed control practices (use of livestock to control some pasture weeds, use of insects or diseases to manage some weeds) and chemical practices.

Use of chemicals is the most effective and convenient way to control and fight weeds with a low economical cost. Herbicides are chemicals specifically designed to kill weeds and they certainly allow economically viable weed control, high yields and increased productivity (Mulwa and Mwanza, 2006). Herbicides can be classified according to their characteristics, including among others application time, crop usage, weed control spectrum and mode of action.

The mode of action of a herbicide is the sequence of events leading to plant death. The site of action, or target site, of the herbicide is the specific metabolic target affected by the herbicide into the plant. Herbicides with similar chemistry can be grouped into “families” and families



sharing the same mode of action into “classes”. Important modes of action include the inhibition of plant amino acid biosynthesis, fatty acids biosynthesis, photosynthesis and pigment synthesis... Tables describing current herbicides’ modes and sites of action are published by the Herbicide Resistance Action Committee (HRAC) and the Weed Science Society of America (WSSA):

<http://www.hracglobal.com/Education/ClassificationofHerbicideSiteofAction.aspx>

<http://wssa.net/wp-content/uploads/WSSA-Mechanism-of-Action.pdf>

Herbicides can be applied at various time points during crop culture, according to crop development or weed growth, depending on their chemical families. Pre-emergence herbicides are applied before planting for control of annual weeds, while post-emergence herbicides are applied after the crop is established.

Herbicides are also classified as contact or systemic. Contact herbicides kill the plant organs to which the chemical was applied and are effective against annual weeds. Systemic herbicides are absorbed by the roots or aerial parts of the plant and translocated through the plant to their site of action. These herbicides are most effective against perennial weeds.

Another important criterion for classification is the degree of selectivity. Selective herbicides kill weeds without damaging the associated crop allowing post-emergence application onto the entire field. Mostly, selectivity is based on the crop’s ability to naturally metabolize the herbicide into non-toxic products. Wheat, for example, can tolerate fenoxaprop-p-ethyl, because it rapidly deactivates the herbicide in non-toxic metabolites, whereas wild oat can only slowly deactivate the herbicide, leading to fenoxaprop-p-ethyl selectivity in wheat fields (Tal *et al.*, 1993). Corn and sorghum are tolerant to atrazine (inhibitor of photosynthesis at photosystem II) because unlike susceptible weeds, they rapidly metabolize the highly phytotoxic compound to a completely non-phytotoxic product (Shimabukuro, 1967).

Non-selective or “total” herbicides possess a broad activity spectrum and thus kill all the vegetation including the crop. They are frequently used to control the vegetation on rail road tracks, public squares, road sides, etc... If a crop can be rendered resistant against a broad spectrum herbicide, they provide a very efficient way for weed control.

Herbicide-tolerant or resistant plants are plants whose growth and development are not damaged or even affected by application of the respective herbicides.



Herbicide tolerance is the natural inherent ability of a species to survive and reproduce after herbicide treatment, implying that there was no selection or genetic manipulation to make the plant tolerant. Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type (Weed Science Society of America, 1998). Resistance may arise naturally during an evolutionary process resulting from the selection of a natural mutation due to the selection pressure caused by the repeated application of herbicides with the same site of action. *Senecio vulgaris* biotypes, for example, are resistant to triazine, a photosystem II (PSII) inhibitor, because of a mutation in the plastoquinone binding D1 protein of PSII (Holt *et al.*, 1981). Resistance may also be induced by genetic engineering or selection of variants produced by tissue culture or mutagenesis. An overview of currently described mechanisms of herbicide resistance is presented in Fig. 1.

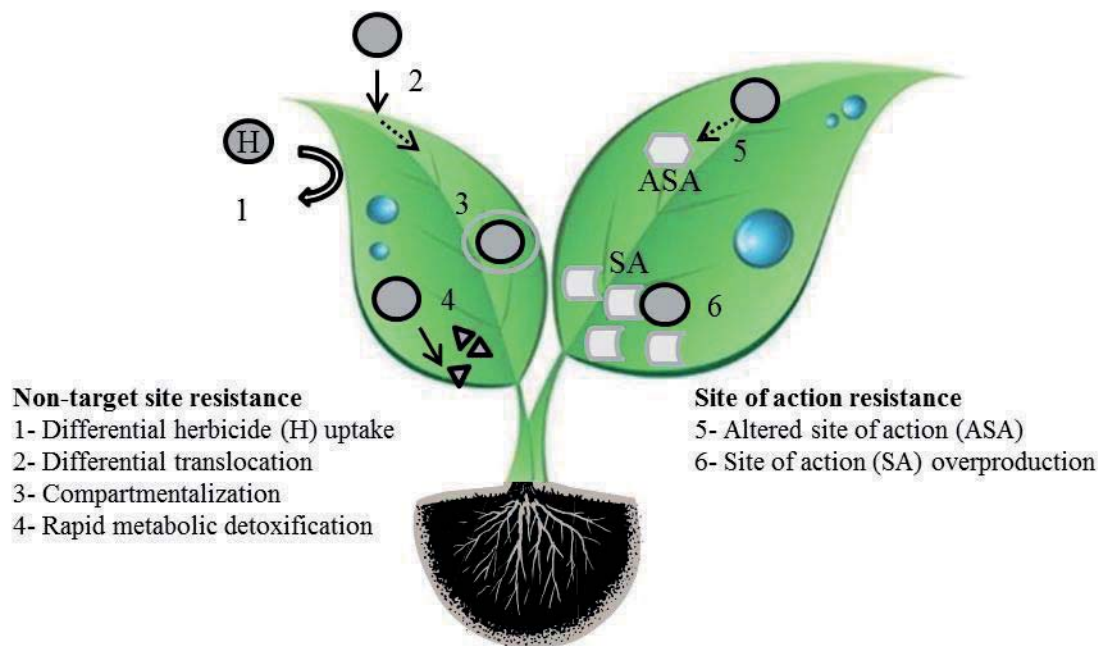


Fig. 1: Main mechanisms of plant herbicide resistance

Since the discovery and analysis of naturally herbicide-tolerant or resistant plants, herbicide-resistant crops have also been conventionally bred or engineered. For instance, various herbicide-resistant canola culture systems are currently available:

- Conventionally-bred herbicide-resistant (HR) canola: triazine resistance was introgressed from a triazine resistant weedy parent (*Brassica rapa*) into canola;



imidazolinone-resistant Clearfield® canola was developed by BASF through somaclonal variation and conventional breeding.

- Genetically-modified HR canola: genes conferring glyphosate or glufosinate-ammonium-resistance have been used to engineer Roundup Ready® canola (Monsanto) and InVigor® canola (Bayer CropScience), respectively.

Coupling the use of non-selective herbicides and herbicide-resistant crops enables farmers to achieve benefits such as control of a broader weed spectrum, reduced crop injury and herbicide drift effects and increase crop management efficiency, simplicity and flexibility (Green and Owen, 2011).

1.2 DEVELOPING A NEW WAY FOR ALFALFA WEED MANAGEMENT

1.2.1 Alfalfa importance

Alfalfa is a perennial cultivated species grown worldwide, cultivation of which began two thousand years ago (Kumar, 2011). Mutations and breeding with wild varieties contributed to evolution of cultivated alfalfa *Medicago sativa subspecies (ssp). sativa*. The common cultivated alfalfa is an autotetraploid plant with $2n = 4x = 32$ and the genomic resources are limited due to the autotetraploidy and the fact that the alfalfa populations are extremely polymorphic. In the same genus, *Medicago truncatula* has been chosen as a model organism for legume biology because of its small diploid genome, its self-fertility, its rapid generation time and its ability to form symbiosis with bacteria or fungi. The genome of *Medicago truncatula* is sequenced (*Medicago truncatula* HapMap Project; Young *et al.*, 2011) and potentially provides insight into the *Medicago sativa* genome because *Medicago sativa* and *Medicago truncatula* share a significant number of conserved sequences (Zhu *et al.*, 2005). Simple sequence repeat (SSR) markers are available for some alfalfa traits (Eujayl *et al.*, 2004; Flajoulot *et al.*, 2005). Recently, single nucleotide polymorphisms (SNPs) have been identified for genotyping and mapping in tetraploid alfalfa (Han *et al.*, 2011).

Alfalfa is primarily used as forage for livestock, as hay or silage and in pasture, due to its high feeding value. The plant contains 15 to 22 % crude protein, minerals and vitamins. It has low



fiber content, resulting in high forage intake (Kumar, 2011). Alfalfa is a well-adapted perennial crop for most environments because of winter hardiness and drought tolerance and is thus often used as a cover or rotational crop. In addition, the well-studied interaction between *Sinorhizobium meliloti* and *Medicago sativa* (among others: Wall and Favelukes, 1991; Timmers *et al.*, 1999; Arrighi *et al.*, 2008; Young *et al.*, 2011) is one of the most efficient nitrogen fixing bacteria/legume associations and is considered to enable an average nitrogen fixation of 450 kg/ha/year (Oliveira *et al.*, 2004). Due to its symbiotic association with nitrogen-fixing bacteria, use of nitrogen fertilizers is not necessary. Its deep root system and its association with mycorrhiza contribute to soil aeration and organic matter content and when plowed down, alfalfa contributes significantly to the nitrogen requirement of following crops (Canadian Food Inspection Agency, 2005). In recent times, alfalfa begins to be used for biofuel production due to its high biomass production, for soil bioremediation, for production of pharmaceuticals and, to a lesser extent, for human food (Kumar, 2011).

Alfalfa is the most cultivated forage crop in the world with more than 35 million hectares. The production is distributed as follows: North America (41 %), Europe (25 %), South America (23 %), Asia (8 %), Africa (2 %) and Oceania (1 %) (Radovic *et al.*, 2009). In terms of area of alfalfa production, the leading countries are the United States of America (9 million ha) and Argentina (6.9 million ha) (Yuegao and Cash, 2009). In the USA, alfalfa harvested crop area is ranking four (2011) before cotton (National Agricultural Statistics Service, 2012). The worldwide alfalfa production was around 436 million tons in 2006 (Food and Agriculture Organization of the United Nations, 2012). Seed yields in many areas average from 800 to 1200 kg/ha/year (Cash and Jingzhong, 2003) with North America being the largest alfalfa seed producing region.

Alfalfa value depends on relative feed value and crude protein content and is thus related to purity and palatability of the harvested forage or seeds. The estimated value of alfalfa hay was \$ 102.50/ton in 1998. In the same year, the total value for alfalfa hay in the US reached \$ 8.1 billion, not including the value of hay mixtures including alfalfa and the value of the final products (dairy or other animal products such as meat or milk). For alfalfa seeds, the estimated value in 1999 was \$ 190/100 pounds (about 45 kg), representing a total value of \$ 218.5 million for alfalfa seeds in the US (Bauchan and Greene, 2000).