Agriculture, Ecosystems and Environment 146 (2012) 153–161

Contents lists available at SciVerse ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Effects of gene flow from IMI resistant sunflower crop to wild *Helianthus annuus* populations

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ARTICLE INFO

Article history:
Received 18 April 2011
Received in revised form 24 October 2011
Accepted 29 October 2011
Available online 24 November 2011

Keywords:
Imidazolinone herbicides
Sunflower
Transference
Dormancy
Fitness

ABSTRACT

Wild sunflower, *H. annuus* ssp. *annuus*, is an invasive species widely distributed in several regions of the world, including central Argentina where it shares a large area with domesticated sunflower. The discovery of imidazolinone resistance in a wild sunflower population allowed the development of Clearfield® technology in cultivated sunflower. This technology was rapidly adopted in Argentina but the trait could possibly be transferred to the naturalized wild populations through natural hybridization. The aim of this study was to evaluate the transfer of IMI resistance to wild sunflower populations and its effect on wild plants' fitness. Plants of five wild populations and their progenies of crosses with an IMI-resistant hybrid were evaluated through a dormancy, herbicide resistance, and SSR markers study. Relative fitness was compared in the five populations, F1s and backcrosses with wild and crop parental plants. Hybridization with an IMI-resistant hybrid did not alter seed dormancy. F1 individuals were more resistant to imazapyr than their wild ancestors but less tolerant than the commercial variety. SSR markers confirmed the transfer of resistance and identified resistant plants within the wild populations. Fitness was reduced in the first generation after crossing but was recovered in the following generations. Thus, to ensure durability and efficiency of Clearfield® technology, management practices like crop rotation and herbicide usage with different modes of action should be considered.

1. Introduction

The remarkable increase of the soybean crop area has displaced sunflower, *Helianthus annuus* L. var. *macrocarpus*, a traditional oil crop in Argentina, toward less fertile areas (de la Vega et al., 2007). The new crop region greatly overlaps the distribution area of a wild naturalized *Helianthus* species, *H. annuus* ssp. *annuus* which has became widespread throughout the country during the last 60 years (Poverene et al., 2008).

The discovery of a wild *H. annuus* population resistant to imidazolinone in Kansas, USA, by natural selection of a mutant after 7 years of imazethapyr treatment (Al-Khatib et al., 1998), allowed the development of the Clearfield® technology. This technology confers tolerance to several imidazolinone (IMI) herbicides and has been incorporated into the crop through conventional breeding techniques. IMI sunflower hybrids have been commercialized in Argentina since 2003 (Zollinger, 2003).

The imidazolinone herbicide family inhibits the acetohydroxyacid synthase enzyme (AHAS, EC 4.1.3.18) also known as acetolactate synthase, which catalyzes the biochemical synthesis of the branched aminoacids, isoleucine, leucine, and valine (Shaner et al., 1984). Herbicides with the same mode of action are broadly utilized because they are effective at low doses, pose low toxicity, and control a wide range of weeds (Tan et al., 2005). The high level of resistance to imidazolinone found in the wild sunflower was attributed to the presence of an altered AHAS form which makes it less sensitive to the herbicide (Al-Khatib et al., 1998).

The IMI resistance is controlled by two genes: a main one Imir1, and a secondary one Imir2 (Bruniard and Miller, 2001). Total resistance is achieved only when both genes are homozygous (Imir1 Imir1 and Imir2 Imir2). A base substitution in the genes encoding AHAS can alter the tolerance to herbicides that inhibit this enzyme (Tan et al., 2005). Kolkman et al. (2004) identified three encoding genes, AHAS1, AHAS2, and AHAS3. They found a mutation in codon 205 (ALA205) of AHAS1 that confers imidazolinone tolerance and designed primers to detect allelic variants in a linked microsatellite. Hence it would be possible to molecularly characterize individuals tolerant to herbicide or not, given that they differ in alleles controlling variants of the AHAS enzyme. A new variant in codon 122 of the AHAS1 gene has been recently induced by mutagenesis in an elite sunflower line (Sala et al., 2008). This variant, known as

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doi:10.1016/j.agee.2011.10.023

CLHA-plus, confers higher tolerance levels and more selectivity to imidazolinone herbicides than the Clearfield® technology (BASF, 2010).

Spontaneous mutations conferring resistance to AHAS inhibitors can rapidly increase their frequency in weedy populations under a high selection pressure due to herbicide usage (Tranel and Wright, 2002). Since the release of the first herbicide inhibiting AHAS, chlorsulfuron in 1982, tolerant weeds to this herbicide group have increased in frequency, reaching the highest rate of increase (Heap, 2010). Given the existence of wild *H. annuus* populations established in the central region of Argentina, and the possibility of giving rise to fertile hybrids with domesticated sunflower (Ureta et al., 2008), the widespread usage of IMI technology could favor the gene transfer of the trait. However, resistance in wild local species has not been reported to date.

Gene transfer of herbicide resistance from crop to wild species could generate noxious weed biotypes difficult to control (Ellstrand, 2003). The main concern is the increasing of wild relative fitness as a consequence of introgressed genes, creating a more invasive weed (Snow and Morán-Palma, 1997; Arriola and Ellstrand, 1997; Ellstrand et al., 1999). Fitness is the potential success of a genotype based on survival and fecundity components, i.e. dormancy, germination, vegetative growth, flowering, pollen and seed production (Jenczewski et al., 2003). Nevertheless, crop trait introgression can generate maladapted hybrids exhibiting low fitness (Stewart et al., 2003) with crop alleles often pervasive in wild populations (Whitton et al., 1997; Hansen et al., 2001; Snow et al., 2001). Thus, it is important to estimate the fitness effects of crop genes introgressed in wild populations or weeds over several generations.

Dormancy is an internal seed condition that prevents germination under appropriate hydric, thermic, and gaseous conditions (Benech-Arnold et al., 2000). This mechanism allows the persistence of invasive species in the seed bank, including wild sunflower (OECD, 2004; Martínez-Ghersa and Ghersa, 2006). Sunflower seeds exhibit a physiological dormancy, where an embryo mechanism hampers germination. Seed pericarp operates in seed dormancy affecting germination and seedling establishment, hindering water absorption and containing chemical inhibitors. Also, it reduces dehydratation and increases its persistence in the soil seed bank (Baskin and Baskin, 1998; Hu et al., 2009). Crop hybridization can reduce dormancy in a wild species (Groot et al., 2003; Bagavathiannan and Van Acker, 2008). In sunflower there is not a unique response to wild-crop gene flow, since dormancy depends on the wild genetic background and the domestic genotype (Snow et al., 1998; Mercer et al., 2006a).

In this study we investigated if IMI sunflower crop traits would persist in wild *H. annuus* populations. Following the successive steps to overcome by introgressed germplasm, dormancy, survival to herbicide selection and fecundity of wild-crop hybrids relative to wild were evaluated. Modification of wild sunflower fitness would indicate a potential environmental impact. Laboratory, greenhouse and experimental field studies were undertaken to address the following questions. (1) Would the IMI trait confer IMI tolerance in wild-crop hybrids? (2) Would crop traits affect seed dormancy in wild-crop hybrids? (3) Would crop traits have an effect on relative fitness of wild-crop hybrids? (4) Would the IMI trait persist in wild populations?

2. Materials and methods

2.1. Plant material

Five accessions of wild *Helianthus annuus* (WILD) representative of invasive populations from the central region of Argentina were evaluated: Río Cuarto (RCU), Colonia Barón (BAR), Adolfo Alsina (AAL), Diamante (DIA), and Las Malvinas (LMA). Achenes were collected in 2002 and stored at room conditions. Before sowing, seed dormancy was broken by maintaining seeds on germination paper in a wet chamber at 5 °C for 1 week (ISTA, 2004). Seedlings were grown for 30 days in the greenhouse at 20–25 °C, and then transplanted at the 4–6 leaf stage to a common garden at the Agronomy Department (S 38°41′38″, W 62°14′53″) Universidad Nacional del Sur, Bahia Blanca, Argentina. The accessions were regenerated by controlled pollination of 20–30 heads covered with paper bags at the R4 stage (Schneiter and Miller, 1981).

Controlled crosses between the wild accessions and the sunflower commercial hybrid DK3880CL (IMI) were made according to Jan and Seiler (2007) to produce F1 progeny. Disk flowers were emasculated in the morning and pollinated with DK3880CL in late afternoon. In the following generation, F1 plants were emasculated and hand-pollinated with the respective wild accession or with DK3880CL to produce backcrosses ($BC1_W$, $BC1_C$). WILD, F1, $BC1_W$, and $BC1_C$ were considered cross types. The five cross types derived of each one of the five wild accessions were considered a family.

The SSR study was done on WILD and F1 from DIA, LMA and RCU accessions and the inbred lines HA89, HAR2, HAR3, HAR5, HA369, and RHA274 (obtained from INTA Balcarce Experimental Station, Argentina) as controls. These inbred lines have been extensively used in sunflower breeding programs in Argentina. DK3880CL and HA89 × DK3880CL progeny were used as herbicide tolerant controls. AAL and BAR were excluded from the SSR analysis because markers gave unexpected results. These two populations are sympatric with *Helianthus petiolaris* populations and the likely interspecific hybridization could have produced chromosomal rearrangements (Rieseberg et al., 1995). As a consequence, in a number of plants the SSR markers were not linked to the IMI trait or either showed new allelic variants.

2.2. Seed germination study

Germination was evaluated in the wild accessions and in their F1s immediately after harvest, after 6 and 12 months of dry storage at 5 °C. Germination using wet paper towels was recorded during 2 weeks in a growth chamber at 20 °C with a neutral photoperiod. To estimate dormancy, a tetrazolium viability test was performed after 15 days on non-germinated seeds (ISTA, 2004).

The experiment was conducted as a randomized complete design with four replicates with experimental units of 25 seeds. Data were analyzed using ANOVA, and prior arcsine transformation. Sources of variation were cross type, family and storage time.

2.3. Herbicide treatment

Two experiments were performed to estimate the response to imazapyr (Clearsol®) in WILD and F1 cross types. Plants were grown up to 2–4 leaf stage in 24 cm × 54 cm plastic trays ($N = 128$). Herbicide was applied with a constant pressure laboratory sprayer with 8001 flat spray tip calibrated to deliver 105 l ha^{-1} at 142 kPa adding 0.05% Canoplus® as a surfactant.

In exp. 1, the five cross types were sprayed with 0, 0.5, 2, and 8× imazapyr (Clearsol®, $X = 80$ g ai ha^{-1}). In exp. 2, the cross types of DIA, LMA, and DK3880CL were evaluated to perform a dose–response curve. In this approach the treatments were 0, 0.06, 0.13, 0.5, 1, 2, 8, and 16×, the normal use rate of the herbicide. Visible injury was estimated 21 days after treatment on a scale ranging from 0 = without damage, 1 = 25% damage, 2 = 50% damage, 3 = 75% damage and 4 = dead apex (Al-Khatib et al., 2000). At this stage plants were dissected into aerial and root parts, and dried at 60 °C for 7 days and weighed. Experiments were conducted as a randomized complete design. Each family was represented