

## Genetic quality control in Clearfield Plus sunflower hybrids

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### ABSTRACT

- Clearfield Plus hybrids carry the *Ahas11-3* allele in homozygous condition or in heterozygous state together with the *Ahas11-1* allele (Imisun). The first hybrids launched to the market belong to this last type because it is easy to obtain them by converting the restorer line to Clearfield Plus and using an Imisun sterile line as the female. However, the use of this type of hybrids might involve at least three different sources of off-types in the final product: a) foreign pollen from non-tolerant sources originating heterozygous *Ahas1-1/ahas1* plants, b) foreign pollen from Imisun hybrids or lines originating homozygous Imisun *Ahas11-1/Ahas11-1* plants, and, c) pollen from spontaneous maintainer plants in the male sterile seed lot, which also originate homozygous Imisun seeds. The new herbicide Clearsol Plus (Imazamox 3.3 % + Imazapyr 1.5 %) produces phytotoxicity symptoms when it is applied on homozygous and heterozygous Imisun plants. For this reason, the exact determination of the number of off-types bearing these genotypes in a seed-lot of a Clearfield Plus hybrid is very important to guarantee quality standards of the final product that is delivered to the farmer. A protocol to do this was developed and in this work we describe it and present its validation.
- Seventy-three lots belonging to two experimental Clearfield Plus hybrids were artificially contaminated with Imisun or Imisun/wild type hybrids to obtain seed contamination levels ranging from 0.2 % to 6.5 %. Contaminant seeds were prepared as a mix consisting of 15% of *Ahas1-1/ahas1* and 85% of *Ahas11-1/Ahas11-1* seeds. Samples of 500 and 2000 seeds from these lots were sown under two different environmental conditions (field and greenhouse), during the winter in Formosa province, Argentina. Clearsol Plus was sprayed at V2-V4 stage of plant development using a 1x field rate. Ten to twelve days after herbicide application plants were visually scored as fully tolerant or non tolerant. Scored percentages of off-types were tested for deviations from known contamination values using a t-test. Phytotoxicity symptoms (deformations, chlorosis, and necrosis) were assessed and rated for each non-tolerant plant. Leaf tissue of each non-tolerant plant and several tolerant plants were sampled for molecular characterization analysis. DNA studies were carried out using allele specific markers previously reported.
- No significant differences were observed between actual and scored percentages of off-types when the evaluations were carried out under greenhouse conditions. However, when considering the data obtained under field conditions, a significative deviation between scored percentages and known contamination values was detected ( $p < 0.001$ ). Assessment of phytotoxicity symptoms allowed us to classify non tolerant plants into two well defined groups. Molecular analysis confirmed that all the plants visually scored as tolerant were of genotype *Ahas11-3/Ahas11-1*, while 99% of the sampled non tolerant plants were devoid of the *Ahas11-3* allele. Non tolerant plants were genotyped as homozygous *Ahas11/Ahas11* and heterozygotes *Ahas11-1/ahas1* in different rates according to the environmental condition during the assessment (86:14 for greenhouse and 59:41 for field conditions). The observed genotypic rate of non-tolerant plants under field conditions was significant different from the original contamination rate ( $p < 0.001$ ). Moreover, there were no clear associations between phenotype clustering of non-tolerant plants and their genotypes at the *Ahas11* locus in either environmental condition during the assessment.
- These results indicate that it is feasible to visually identify non-tolerant plants in batches of Clearfield Plus hybrid seeds after the application of Clearsol Plus herbicide, and, thereby to establish contamination levels.
- Environmental conditions during the assessment, however, can affect the accuracy of the method. In fact, assessment under greenhouse conditions accurately predicts the actual levels of contamination of the evaluated seed lots and also, the genotypic structure of the mix used to contaminate them. Under field conditions, however, accuracy of the method decreased because low stringent conditions did not permit to clearly identify certain contaminating genotypes.
- Genetic quality control of Clearfield Plus hybrids can be assessed under off-season conditions to assure the quality of the commercial hybrids and their parental lines. Environmental stringency should be adjusted by herbicide dose under certain situations in order to achieve accurate determinations.

**Keywords:** Seed production, quality control, imidazolinone.

## INTRODUCTION

Weeds compete with sunflower for moisture, nutrients, and depending on species for light and space. Weed competition causes substantial yield losses in sunflower, with reports ranging from 20 to 70%. The amount of yield reduction varies depending on the weed species, weed density, time of weed and crop emergence, climatic conditions, and type of soil. Herbicides are the most desirable method for weed control; however, the availability of selective herbicides for the sunflower crop is quite limited and, due to the high cost of herbicide registration, new molecules of herbicides are unlikely to be specifically developed for weed control in this crop. For this reason, gene discovery and trait development for herbicide resistance in sunflower is one of the most important issues in raising the productivity and the competitive ability of this crop in the near future (Sala et al., 2012).

The imidazolinone family of herbicides control weeds by inhibiting a key enzyme in the branched chain amino acid biosynthetic pathway, acetohydroxyacid synthase (AHAS; EC 4.1.3.18) also known as acetolactate synthase (ALS) (Shaner et al., 1984; Tan et al., 2005). During the past decade two technologies of weed control that make use of imidazolinones and different mutations at the *Ahas11* locus were developed for sunflower. The Clearfield® system is based on two genes (*Ahas11-1* and an enhancer, Tan et al., 2005), and the Clearfield Plus system, based on the allele *Ahas11-3* alone or in combination with *Ahas11-1* (Sala et al., 2008 a,b,c).

Clearfield Plus hybrids might carry the *Ahas11-3* allele in homozygous condition or in heterozygous state together with the *Ahas11-1* allele (Imisun). The first hybrids launched to the market belong to this last type because it is easy to obtain them by converting the restorer line to Clearfield Plus and using an Imisun sterile line as the female. However, the use of this type of hybrids might involve at least three different sources of off-types in the final product: a) foreign pollen from non-tolerant sources originating heterozygous *Ahas11-1/ahas11* plants, b) foreign pollen from Imisun hybrids or lines originating homozygous Imisun *Ahas11-1/Ahas11-1* plants, and, c) pollen from spontaneous maintainer plants in the male sterile seed lot, which also originate homozygous Imisun seeds.

The new herbicide Clearsol Plus (Imazamox 3.3 % + Imazapyr 1.5 %) produces phytotoxicity symptoms when it is applied on homozygous and heterozygous Imisun plants, because *Ahas11-1* confers only a moderate tolerance to imidazolinones when compared with *Ahas11-3* (Sala et al., 2008b). Using this herbicide it is possible to determine the number of off –types plants bearing the above mentioned genotypes in a seed-lot of a Clearfield Plus hybrid, which is very important to guarantee quality standards of the final product that is delivered to the farmer. A protocol to do this was developed and in this work it is described and validated.

## MATERIALS AND METHODS

Seventy-three lots belonging to two experimental Clearfield Plus hybrids were artificially contaminated with Imisun or Imisun/wild type hybrids to obtain seed contamination levels ranging from 0.2 % to 6.5 %. To do this, 3 kg of seeds from 40 seed lots of Paraiso 1000 CLPlus and 33 seed lots of Paraiso 1001 CLPlus were contaminated with a mix of seeds consisting of 15% of *Ahas11-1/ahas11* and 85% of *Ahas11-1/Ahas11-1* genotypes. Contamination levels were produced based on a weight/weight basis.

Samples of 500 and 2000 seeds from these lots were sown under two different environmental conditions (field and greenhouse), during the winter season in Formosa province, Argentina. Clearsol Plus was sprayed at V2-V4 stage of plant development (Schneider and Miller, 1981) using a 1x field rate (in a volume of 200 l ha<sup>-1</sup>). Ten to twelve days after herbicide application plants were visually scored as fully tolerant or non-tolerant based on the injury induced by the herbicide treatment. Plants were scored phenotypically using a Phytotoxicity Index (PI). PI is a phenotypic scale from 0 to 9 that was assessed for each plant by visual inspection (Sala et al., 2008b) and in this way phytotoxicity symptoms (deformations, chlorosis, and necrosis) were assessed and rated for each non-tolerant plant.

Significance of differences among observed and actual number of off-types plants was conducted using a t-test analysis. Observed percentages of off-types were plotted against actual percentages of off-types for each off-type class (total, Imisun homozygous, Imisun/wild type heterozygous). Simple regression correlation analysis were performed.

Leaf tissue of each non-tolerant plant and five tolerant plants per lot were sampled for molecular characterization analysis. DNA studies were carried out using allele specific markers as previously reported (Bulos et al., 2010).

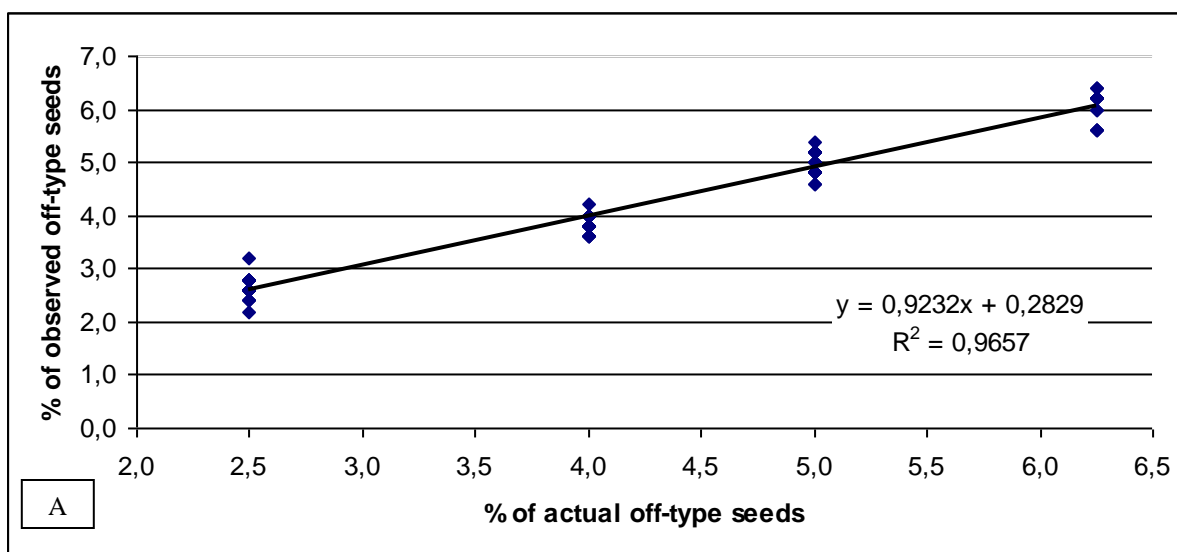
## RESULTS

No significant differences were observed between actual and expected percentages of off-types when the evaluations were carried out under greenhouse conditions. However, when considering the data obtained under field conditions, a significant deviation between scored percentages and known contamination values was detected ( $p < 0.0001$ ) (Table 1).

Table 1. Summary statistics of contamination values in sunflower seed lots scored under two different environmental conditions

	Mean number of off-types	Range	SD	Difference with expected values		
				Mean	p	CI 95%
Greenhouse (N=500)	21.1	11 to 32	6.21	0.222	0.134	-0.071 to 0.516
Field (N=2000)	70.6	27 to 109	24.31	14.67	<0.0001	13.28 to 16.06

Assessment of phytotoxicity symptoms allowed us to classify non-tolerant plants into two well defined groups. Under greenhouse conditions off-type seeds belonging to Imisun or Imisun/wild type were accurately detected and no significant differences were observed between actual and scored percentages of each kind of off-type (Fig. 1). A high correlation ( $b=0.923$ ;  $R^2=0.966$ ) was obtained between the actual and the observed percentage of off-type plants. These off-type plants are composed by Imisun homozygous plants and Imisun/Wild type heterozygous plants. For the first class of off-types a high correlation were observed ( $b=0.954$ ;  $R^2=0.962$ ), however, for the second class, due to the smaller sample size, a lower correlation was observed ( $b=0.751$ ;  $R^2=0.54$ ).



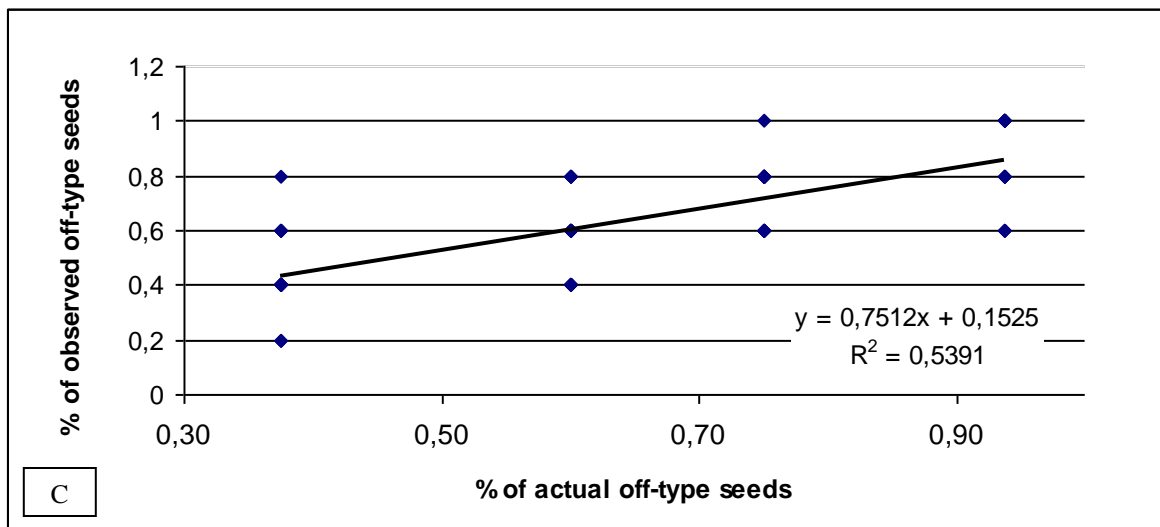
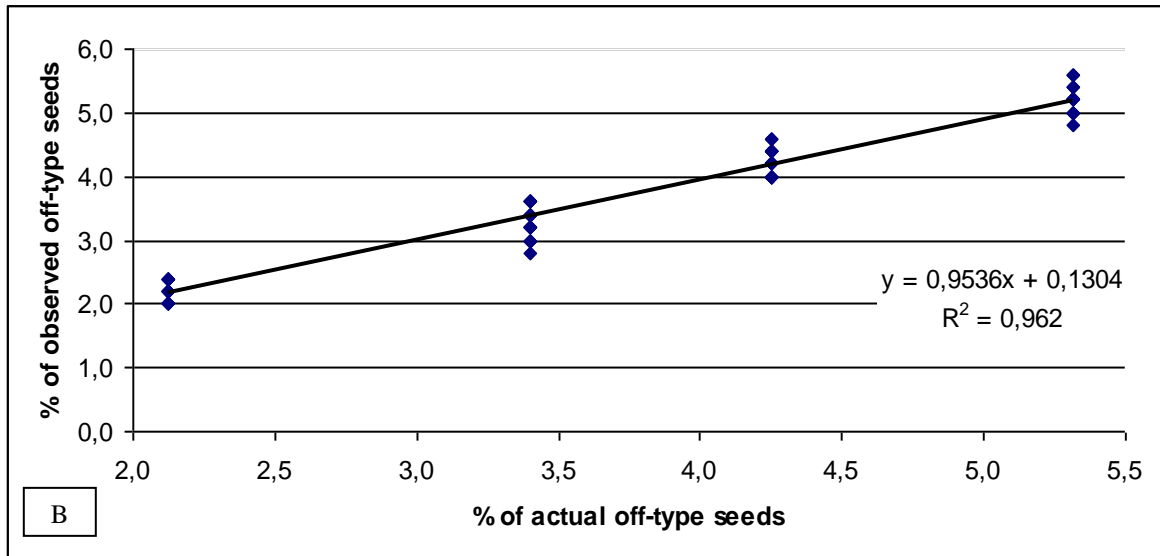


Fig. 1. Association between actual and observed percentages of off-types: A) total, B) Imisun homozygous and C) Imisun/wild type.

Under field conditions off-type plants belonging to Imisun homozygous type were not accurately detected. On the other hand, Imisun/wild type plants were detected without any difficulty. A lower correlation ( $b=0.897$ ;  $R^2=0.952$ ) was obtained between the actual and the observed percentage of off-type plants. This was mainly produced because of the lower correlation observed for the actual and the observed percentage of Imisun homozygous off-type plants ( $b=0.877$ ;  $R^2=0.936$ ). In this case a high correlation was observed for Imisun/wild type off-type plants ( $b=1.014$ ;  $R^2=0.952$ ).

Molecular analysis of the sampled plants considered as tolerant confirmed that these plants were correctly scored and belongs to the genotype *Ahas11-3/Ahas11-1*, while 99% of the sampled non tolerant plants were devoid of the *Ahas11-3* allele. Non tolerant plants were genotyped as homozygous *Ahas11/Ahas11* and heterozygotes *Ahas11-1/ahas11* in different rates according to the environmental condition during the assessment (86:14 for greenhouse and 59:41 for field conditions). The observed genotypic rate of non-tolerant plants under field conditions was again significantly different from the original contamination rate ( $p<0.001$ ). Moreover, there

were no clear associations between phenotype clustering of non-tolerant plants and their genotypes at the *Ahas11* locus in either environmental condition during the assessment.

## DISCUSSION

In many crops, where herbicide resistant traits have been developed many years ago, different bioassays have been described to detect herbicide resistant traits (Torres et al., 2003; Tillmann et al., 2004; Tripathi, 2005). In sunflower, Breccia et al. (2011) described a rapid test for detection of imidazolinone resistant plants based on a germination test using imazapyr, that was mainly developed to assist breeding programs selecting for *Ahas11-1* alleles.

Quality control of Clearfield Plus hybrids imposes a more complex scenario because of the multiallelic nature of *Ahas11* locus for herbicide resistance. The challenge now, is not only to detect susceptible plants in a herbicide tolerant seed lot, but also to detect different herbicide resistant allele combinations that were now considered as off types.

The results obtained here indicate that it is feasible to visually identify non-tolerant plants in batches of Clearfield Plus hybrid seeds after the application of Clearsol Plus herbicide, and, thereby to establish contamination levels.

Environmental conditions during the assessment, however, can affect the accuracy of the method. In fact, assessment under greenhouse conditions accurately predicts the actual levels of contamination of the evaluated seed lots and also, the genotypic structure of the mix used to contaminate them. Under field conditions, in the other hand, accuracy of the method decreased because the low stringent conditions imposed did not permit to clearly identify certain contaminating genotypes. In particular, homozygous Imisun plants showed almost a tolerant behaviour when applied with 1x of Clearsol Plus herbicide under field condition, which implied a difficult in phenotypic injury characterization. Almost half of Imisun homozygous plants were scored as types tolerant plants.

Based on these results, genetic quality control of Clearfield Plus hybrids can be assessed under off-season conditions to assure the quality of the commercial hybrids and their parental lines. Environmental stringency should be adjusted by herbicide dose under certain situations in order to achieve accurate determinations.

Due to the rapid adoption of Clearfield Plus technology and the greenhouse area needed to cope with this type of genetic quality control analysis, high throughput Real-Time PCR assays developed to detect off-type alleles in *Ahas11* locus are under development.

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