

Soil-less bioassay for the identification of plants carrying different imidazolinone-tolerant alleles in sunflower

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ABSTRACT

- The multiallelic *Ahas11* locus of sunflower determines the level of imidazolinone (IMI) tolerance in this species. *Ahas11-1* and *Ahas11-3* are IMI tolerance alleles at this locus controlling the traits known as Imisun and CLPlus respectively. *Ahas11-1* harbours a C-to-T mutation in codon 205 which confers a moderate tolerance to imidazolinones, while *Ahas11-3* has a G-to-A mutation in codon 122 which confers high levels of tolerance to imidazolinones. Both alleles are being used for the production of sunflower hybrids tolerant to imidazolinones. Rapid and efficient tests for early screening of herbicide tolerance are convenient alternatives to field screening methods. The objective of this study was to evaluate the growth response of several genotypes at the *Ahas11* locus differing in IMI tolerance using a soil-less bioassay.
- Six sunflower genotypes containing different combinations of alleles at the *Ahas11* locus were used in this study: homozygous CLPlus (*Ahas11-3/Ahas11-3*), heterozygous Imisun/CLPlus (*Ahas11-1/Ahas11-3*), CLPlus heterozygous (*Ahas11-3/ahas11*), Imisun homozygous (*Ahas11-1/Ahas11-1*), Imisun heterozygous (*Ahas11-1/ahas11*), and susceptible wildtype (*ahas11/ahas11*). Achenes were planted on plastic pots filled with commercial perlite. The pots were placed in plastic trays and watered by capillarity with a nutritive solution consisting of Murashige and Skoog's medium (1.1 g l⁻¹) and commercial herbicide Clearsol Plus® (BASF). Five concentrations of herbicide were used: 1 – 5 – 10 – 50 – 100 µM. The nutritive solution without herbicide was used as control. Pots were incubated for 7 days at 25 ± 2 °C with a 16h photoperiod (100 µmol m⁻² s⁻¹). The experimental design was a randomized block design with four replications and each replication consisted of 12 plants (total number of plants = 1728). After the incubation period plantlets were collected and scanned into digital images. Hypocotyl length (HL), primary root length (RL) and the longest lateral root length (LL) were measured from scanned images using ImageJ 1.44. Normality of the empirical distribution of each variable was assessed by the Shapiro-Wilk W test statistic. Homogeneity of variance was evaluated using the Levene's test on absolute residuals. Within a genotype, treatments means were compared using Tukey's multiple-comparison test.
- Sunflower plantlets reached the VE stage 7 days after planting. All genotypes showed a reduced growth when exposed to herbicide treatments but in different levels according to the alleles combined in the *Ahas11* locus. Plantlets of the susceptible genotype became stunted when exposed to herbicide treatments. Primary roots of susceptible plants did not exceed 10 to 15 mm in length and did not develop lateral roots. Among the measured variables, LL -which is associated with the reduction of lateral root growth-, was useful in discriminating among the evaluated genotypes. Homozygous CLPlus plants showed the highest level of tolerance since LL for this genotype was only significantly affected at herbicide doses of 50 µM or higher. Imisun/CLPlus heterozygotes showed a similar response as homozygous CLPlus plants, but they showed a significant reduction for LL at 10 µM dose. Homozygous Imisun, heterozygous CLPlus, and heterozygous Imisun genotypes displayed the same behavior showing the lowest level of root growth among the tolerant genotypes since LL in these genotypes was diminished at 1 µM.
- Root growth response to increased doses of IMI for the evaluated genotypes showed the same pattern of response than the above-ground biomass accumulation after IMI spraying over the leaves reported previously. Moreover, dominance relationships among different members of the *Ahas11* locus for root growth are also conserved. Visual inspection of root development could be useful criteria to distinguish among susceptible plants and those expressing different IMI tolerance alleles.
- The seed germination bioassay described in this study could allow the implementation of fast, reliable and cost-effective diagnosis method that could be useful to assist breeding programs. From another technological perspective, this method permit to differentiate the tolerance endowed by different *Ahas11* genotypes to several AHAS inhibitor molecules present as residues in the soil.

Key words: imazamox - imazapyr - herbicide tolerance- root growth

INTRODUCTION

Imidazolinone herbicides control a wide spectrum of grass and broadleaf weeds at low application rates and have favourable environmental properties compared to other chemistries (Tan et al., 2005). This family of herbicides is specific and potent inhibitors of acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS), which is the first common enzyme in the biosynthetic pathway of branched-chain amino acid (Shaner et al., 1984). Several variant AHAS genes conferring imidazolinone tolerance were discovered in several plants through mutagenesis and selection, and were used to create imidazolinone-tolerant crops. In sunflower the multiallelic *Ahas11* locus determines the level of imidazolinone (IMI) tolerance in this species. *Ahas11-1* and *Ahas11-3* are IMI tolerance alleles at this locus controlling the traits known as Imisun and CLPlus, respectively. *Ahas11-1* (also known as *I_{mr1}* or *Ar_{pur}*, Bruniard and Miller, 2001; Kolkman et al., 2004) harbours a C-to-T mutation in codon 205 and confers a moderate tolerance to imidazolinones. This trait was discovered in a wild sunflower population in Kansas, USA (Al-Khatib et al., 1998) and was the first commercial imidazolinone tolerance trait in this species. The second allele *Ahas11-3* or CLPlus has a G-to-A mutation in codon 122 which confers high levels of tolerance to imidazolinones. This tolerant trait was developed by seed mutagenesis and selection with imazapyr (Sala et al., 2008a).

To date both alleles are being used for the production of sunflower hybrids tolerant to imidazolinones. Phenotypic identification of imidazolinone tolerance in the field is time consuming and requires a large amount of resources and space. Thus the development of efficient and reliable diagnostic tests for early screening of this trait is needed. Previous studies showed that seed germination bioassays could have utility for screening at early stages of development (Vega et al., 2009; Breccia et al., 2011). The objective of this study was to evaluate the growth response of several genotypes at the *Ahas11* locus differing in IMI tolerance using a soil-less bioassay.

MATERIALS AND METHODS

Six sunflower genotypes containing different combinations of alleles at the *Ahas11* locus were used in this study: homozygous CLPlus (*Ahas11-3/Ahas11-3*), heterozygous Imisun/CLPlus (*Ahas11-1/Ahas11-3*), CLPlus heterozygous (*Ahas11-3/ahas11*), Imisun homozygous (*Ahas11-1/Ahas11-1*), Imisun heterozygous (*Ahas11-1/ahas11*), and susceptible wildtype (*ahas11/ahas11*).

Achenes were planted on plastic pots (4 cm wide, 5.5 cm tall) filled with commercial perlite (Multiquim SRL, 3-8 mm, Argentina). The pots were placed in plastic trays (6 pots by tray) and watered by capillarity with a nutritive solution consisting of Murashige and Skoog's (1962) medium (1.1 g l⁻¹) and commercial herbicide Clearsol Plus® (BASF) (a.i. imazamox/imazapir 33 g l⁻¹ and 15 g l⁻¹ respectively). Five concentrations of herbicide were used: 1 – 5 – 10 – 50 – 100 µM. The nutritive solution without herbicide was used as control. Pots were incubated for 7 days at 25 ± 2 °C with a 16h photoperiod (100 µmol m⁻² s⁻¹). The first 3 days each tray containing 6 pots was sealed in a polyethylene bag, which was removed on the fourth day. The trays were rotated every day to minimize effects of local variations in the growth chamber. The experimental design was a randomized block design with four replications and each replication consisted of 12 plants (total number of plants = 1728). After the incubation period plantlets were collected and their roots washed to remove perlite debris. Hypocotyl length (HL), primary root length (RL) and the longest lateral root length (LL) were measured from scanned images using ImageJ 1.44 (Abramoff et al., 2004).

Data were analyzed by analysis of variance; normality of the empirical distribution of each variable was assessed by the Shapiro-Wilk W test statistic. Homogeneity of variance was evaluated using the Levene's test on absolute residuals. Within a genotype, treatments means were compared using Tukey's multiple-comparison test. Statistical analyses were performed using R software, version 2.12.1 (R Development Core Team, 2010).

RESULTS

Sunflower plantlets reached the VE stage (Schneiter and Miller, 1981) 7 days after planting. All genotypes showed a reduced growth when exposed to herbicide treatments but in different levels according to the alleles combined in the *Ahas11* locus. From the variables analyzed those related to root growth (RL and LL) were the most informative. Figure 1 shows roots from the control and 10 µM treated plants obtained after 7 days incubation period. Plantlets of the susceptible genotype became stunted when exposed to herbicide treatments. Primary roots of susceptible plants did not exceed 10 to 15 mm in length and did not develop lateral roots. At this herbicide concentration CLPlus plants carrying the *Ahas11-3*

allele in homozygous state developed a complete root system and did not show significant differences with respect to control plants. CLPlus/Imisun plants showed a slightly reduction in development of lateral roots however primary root length was not affected. Significant reduction in both primary and lateral roots was observed for all genotypes carrying the Imisun allele in homozygous or heterozygous state and for the CLPlus heterozygous.

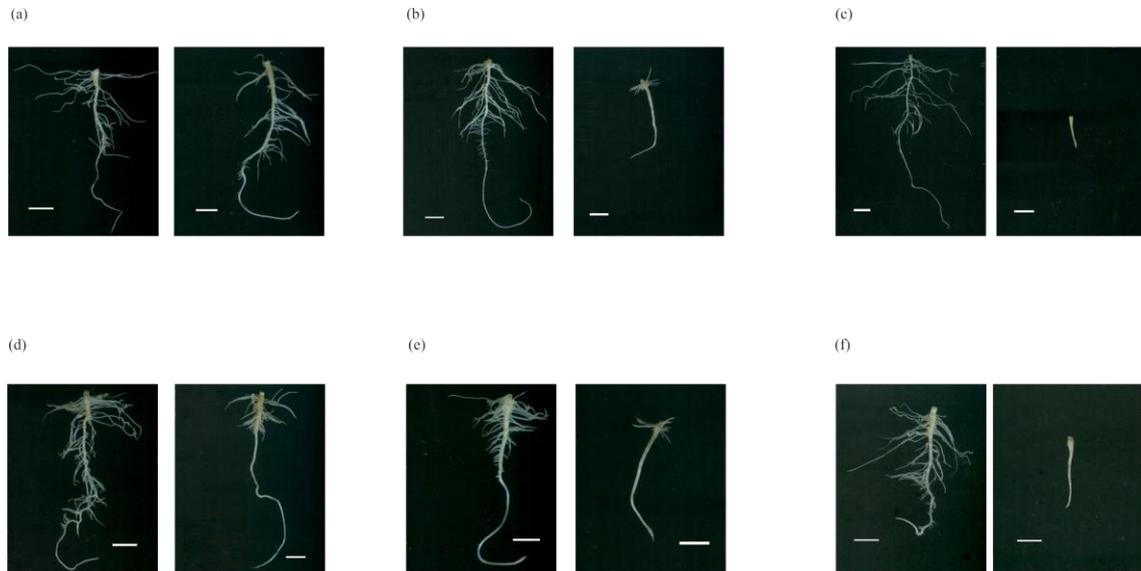


Fig 1. Roots from the plants obtained after 7 days incubation period. Roots from control plants (left) and roots from plants exposed to 10 μM commercial herbicide Clearsol Plus® (right) for the genotypes: (a) CLPlus homozygous (*Ahas11-3/Ahas11-3*) (b) Imisun homozygous (*Ahas11-1/Ahas11-1*) (c) Susceptible (*ahas11/ahas11*) (d) Imisun/CLPlus (*Ahas11-1/Ahas11-3*) (e) CLPlus heterozygous (*Ahas11-3/ahas11*) (f) Imisun heterozygous (*Ahas11-1/ahas11*). Bar= 1 cm

Among the measured variables, LL -which is associated with the reduction of lateral root growth-, was useful in discriminating among the evaluated genotypes (Fig. 2).

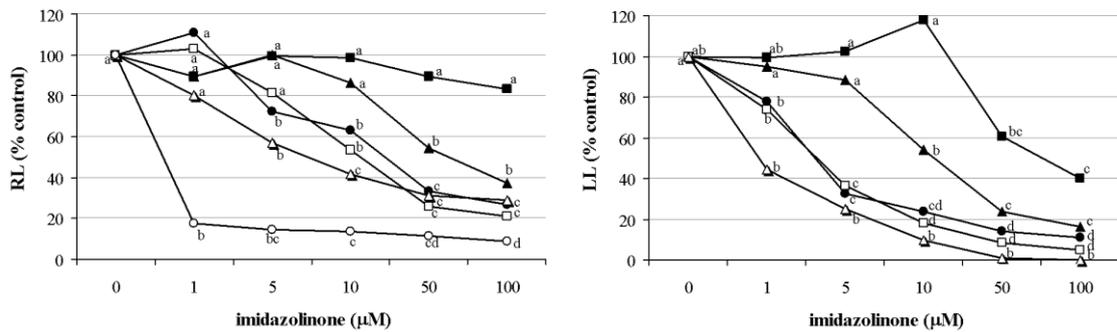


Fig. 2. Effect of commercial herbicide Clearsol Plus® on primary root length (RL) and the longest lateral root length (LL). Values in the plot are the means of each genotype expressed as percentage of the control. Within a genotype, means with the different letter are significantly different ($\alpha=0.05$). CLPlus homozygous *Ahas11-3/Ahas11-3* (□□■□□), Imisun homozygous *Ahas11-1/Ahas11-1* (□□●□□), Imisun/CLPlus *Ahas11-1/Ahas11-3* (□□▲□□), CLPlus heterozygous *Ahas11-3/ahas11* (□□□□□), Imisun heterozygous *Ahas11-1/ahas11* (□□△□□), and susceptible genotype *ahas11/ahas11* (□□○□□). LL was not recorded for susceptible genotype since plantlets did not develop lateral roots.

Homozygous CLPlus plants showed the highest level of tolerance since LL for this genotype was only significantly affected at herbicide doses of 50 μ M or higher. Imisun/CLPlus heterozygotes showed a similar response as homozygous CLPlus plants, but they showed a significant reduction for LL at 10 μ M dose. Homozygous Imisun, heterozygous CLPlus, and heterozygous Imisun genotypes displayed the same behavior showing the lowest level of root growth among the tolerant genotypes since LL in these genotypes was diminished at 1 μ M.

DISCUSSION

Root growth response to increased doses of IMI for the evaluated genotypes showed the same pattern of response than the above-ground biomass accumulation after IMI spraying over the leaves reported previously (Sala et al., 2011). Moreover, dominance relationships among different members of the *Ahas11* locus for root growth are also conserved. Under the conditions of this experiment the AHAS tolerant alleles *Ahas11-1* and *Ahas11-3* showed differences in their response to herbicide, either in homozygous or heterozygous state. Consistent with previous results obtained in field assays (Sala et al., 2008b) genotypes carrying the *Ahas11-3* allele are more tolerant than those carrying the *Ahas11-1* allele after herbicide application.

An interesting result from the present study is that visual inspection of root development could be useful criteria to distinguish among susceptible plants and those expressing different IMI tolerance alleles. Sunflower root growth was defined as a parameter that responds to ALS inhibitors in a sensitive manner (Hernández Sevillano et al., 2001). As a result of limitation of the availability of branched-chain amino acids ALS-inhibitors causes inhibition of root growth predominantly by stopping cell cycle progression in G₁ and G₂ (Rost and Reynolds, 1985). Several studies in other species showed that IMI herbicides damaged root growth at first and finally led to phytotoxic symptoms such as chlorosis of leaves and reduced plant growth (Zhou et al., 2009).

The bioassay described in this study could be an attractive tool for early selection of the herbicide tolerance because of the large amount of individuals screened in a limited space. Furthermore, it does not require as much infrastructure as whole-plant bioassays and can be completed in a shorter period of time. As it was previously observed, this protocol for early screening imidazolinone tolerance in sunflower is simple, accurate and does not require skilled labor (Vega et al., 2009; Breccia et al., 2011). Therefore it could allow the implementation of fast, reliable and cost-effective diagnosis method that could be useful to assist breeding programs.

Practical implications of these results are important in another technological area. It is known that sunflower lines developed to tolerate some AHAS-inhibiting herbicides are susceptible to foliar applications and, in many cases, to soil residues of other AHAS-inhibiting herbicides (Howatt and Endres, 2006). In this sense, the method described in this paper permits to test in a fast and reliable way the pattern of tolerance/susceptibility of any *Ahas* mutation of sunflower to several AHAS inhibitors that can be present as soil residues from the fallow or the previous crop. By this way, a detailed cross-tolerance database can be established for every tolerant mutation of sunflower to assist in crop and weed management.

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