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# Immature Embryo Culture for Early Screening of Imidazolinone Resistance in Sunflower

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## ABSTRACT

The development of imidazolinone-resistant sunflower cultivars represents a great advantage in controlling weeds. This type of herbicide resistance can only be achieved with homozygosis of two resistant genes; hence breeding for this trait is time-consuming. The objectives of this study were to develop plantlets from immature sunflower embryos to evaluate imazethapyr resistance. Three methods were used, an *in vitro* technique and two non-sterile techniques (dish and pot assays). Three genotypes differing in imidazolinone resistance were evaluated for germination and plantlet developmental variables. Immature embryos successfully germinated in the *in vitro* culture and the pot germination techniques having similar efficiency. Given the simplicity of the pot assay, plantlets from pot germination were used for screening herbicide resistance at four different doses: 2.5, 5, 7.5 and 10 µM. Susceptible plants showed chlorosis and arrested growth, mainly when exposed to 7.5 and 10 µM of imazethapyr. Surviving plants were transferred to pots and placed in a greenhouse and successfully completed their lifecycle. The techniques described herein would allow selecting sunflower inbreds for imidazolinone resistance when shortening breeding cycle.

**Keywords:** *Helianthus annuus* L., herbicide resistance, *in vitro* culture, pot germination
**Abbreviation: ALS**, acetolactate synthase

## INTRODUCTION

Weed competition causes substantial yield loss in sunflower (*Helianthus annuus* L.). Competition experiments showed that weeded conditions produced a 20 to 53% loss in yield compared to weed-free conditions (Blamey and Zollinger 1997). Genes for resistance to acetolactate synthase (ALS)-inhibiting herbicides in sunflower have been introgressed from resistant wild populations (ANN-PUR and ANN-KAN) into elite inbred lines for the purpose of developing herbicide resistant cultivars and hybrids (Al-Khatib *et al.* 1998; Miller and Al-Khatib 2002).

Bruniard and Miller (2001) proposed a digenic model for the inheritance of imidazolinone resistance. In this model, a major gene ($Imr_1$) has a semidominant type of gene action and a second gene ($Imr_2$) acts as modifier when the major gene is present. According to these authors, complete resistance in sunflower can only be achieved with homozygosity of both resistant genes ($Imr_1Imr_1Imr_2Imr_2$) in an inbred line or hybrid.

The development of resistant elite inbred lines to further inclusion in hybrid programs involves introgression of the resistance genes into elite germplasm by backcrossing. Besides being a time-consuming process, it also implies one or more generations of self pollination and progeny testing to correctly classify and select the resistant phenotypes (Bruniard and Miller 2001). Operationally, identification of imidazolinone resistant genotypes involves applying herbicide to plants grown in the field or greenhouse, being a time-consuming and costly task. Initially, a laboratory technique that allows early screening resistant genotypes would be a useful tool to help reducing time and resources when breeding for imidazolinone resistance.

Embryo culture is one of the earliest *in vitro* culture methodologies applied to practical problems and has proven of greatest value to breeders (Bridgen 1994). Its major application in sunflower plant breeding has been for overcoming incompatibility in interspecific crosses (Chandler and Beard 1983; Denat *et al.* 1991; Kräuter *et al.* 1991; Sukno *et al.* 1999). Immature embryo culture has also been a technique extensively used for shortening the breeding cycle in this species, allowing four to five generations per year (Plotnikov 1983; Alissa *et al.* 1986; Azpiroz *et al.* 1987; Jambhulkar 1995). In contrast, non-sterile germination techniques represent a simpler and cheaper alternative to *in vitro* culture of immature sunflower embryos (Paul and Barthou 1994; Torresán *et al.* 1996).

Culture of immature embryos for rapid fixation of inbred lines will be of particular interest only if selection can be achieved at the same time. Given that breeding for imidazolinone resistance requires a considerable amount of time and resources, culture of immature embryos followed by selection of herbicide-resistant plantlets could be useful in saving resources and shortening the cycle of development of new varieties.

The present study aimed to develop an efficient method to select imidazolinone-resistant genotypes while shortening breeding cycle. The objectives were to a) compare three different techniques for obtaining plantlets from immature sunflower embryos and b) evaluate different doses of imazethapyr for the selection of genotypes.

## MATERIALS AND METHODS

### Plant material

Three sunflower inbred lines were used in this study: HA425B, 1058-1 and HA89B, which were described as imidazolinone resistant ($Imr_1Imr_1Imr_2Imr_2$), intermediate ($Imr_1Imr_1imr_2imr_2$) and susceptible ($imr_1imr_1imr_2imr_2$), respectively (Bruniard 2001; Bruniard

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and Miller 2001). Immature seeds were collected 15 days after pollination (Zorzoli *et al.* 1994).

## Immature embryo culture

Three different techniques for developing sunflower plantlets from immature embryos were evaluated: an *in vitro* and two non-sterile techniques, namely dish and pot germination.

***In vitro* germination:** *In vitro* germination of immature embryos was performed according to Zorzoli *et al.* (1994). Immature achenes were surface-sterilized by immersion in 70% ethanol for 1 s and in 3% solution of sodium hypochlorite for 10 min and then rinsed three times in sterile distilled water. The pericarps were removed in sterile conditions and the embryos were germinated on hormone-free half strength Murashige and Skoog (MS) (1962) medium supplemented with 20 $gl^{-1}$ of sucrose, 9 $gl^{-1}$ agar at pH 6.00. Embryos were placed in a test tube containing 10 ml of solid medium. Two embryo manipulations were evaluated: intact embryos and cut embryos; the latter being embryos detached of their distal third cotyledonary end. Cultures were incubated in a growth chamber at 25 ± 2°C with a 12-h photoperiod (50 µmol $m^{-2}$ $s^{-1}$) for 15 days.

**Non-sterile germination techniques:** Immature achenes were surface-sterilized as described above and then rinsed three times in distilled water. The pericarps and seminal membranes were removed from intact and cut embryos. Both embryo manipulations were incubated under the same conditions described for the *in vitro* technique. Two non-sterile assays were evaluated: dish germination and pot germination technique. The dish germination technique used in this study was adapted from Paul and Barthou (1994) as follows: i) embryos were placed in each 10-cm Petri dish on filter paper and cotton soaked with nutritive solution (25% MS), ii) five days after sowing, germinated embryos were transferred to plastic trays filled with sand and watered with nutritive solution. The plantlets were maintained in growth chamber for 10 days.

**The pot germination technique** consisted in sowing immature embryos on plastic pots (4 cm diameter × 5.5 cm tall) filled with sand. The pots were placed in plastic trays (30 pots per tray) and watered by capillarity with nutritive solution. Embryos were incubated for 15 days.

## Experimental design and statistical analysis

A completely randomized design with three replications was used to evaluate the effect of the technique, genotype and embryo manipulation. For *in vitro* experiments, each replication consisted of 10 embryos arranged in 10 test tubes with one embryo each. For dish experiments, each replication consisted of a Petri dish with 10 embryos and for pot germination technique, each replication consisted in 10 pots with one embryo each (total number of embryos = 540).

After the incubation period, embryos having green cotyledons were recorded as germinated and included in the percentage of germinated embryos (GE). Rooted (RE) and elongated embryos (EE) were determined as embryos developing a 1 cm root length and 1 cm hypocotyls, respectively, and represented as percentage over the total number of embryos planted. Individuals at the two-leaf stage were scored for the percentage of plants (PL). No plantlets were obtained from the dish germination technique so results were not given in Tables. Differences between embryo treatments were analyzed by a non-parametric $\chi^2$ test. To assess independence among genotype, embryo manipulation and percentage of plants, a non-parametric test of independence was conducted (G-test) (Sokal and Rolhf 1969).

## Screening for imazethapyr resistance

Sunflower plantlets developed from immature embryo culture were treated with imazethapyr (ammonium 5-ethyl-2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl] nicotinate) in order to screen imidazolinone resistance. At the two-leaf stage, 50 plantlets of each genotype developed from intact immature embryos from the pot germination assay were watered by capillarity with nutritive solution and herbicide. The doses of imazethapyr used were 0 (untreated control) 2.5, 5, 7.5 and 10 µM. Pots were incubated under the same conditions described for *in vitro* culture. For each genotype records of visual symptoms on plantlets were collected. After 8 days, surviving plants were transferred to 10-liter pots filled with a mixture of sterile soil and commercial perlite (2:1) and placed in a greenhouse.

## RESULTS

Immature embryos successfully germinated in the *in vitro* culture and in the non-sterile pot technique developing normal plantlets once the incubation period ended (**Fig. 1**).

No plantlets were obtained from the dish germination technique. Even though the cotyledon surface in contact with the filter became green and increased its size, most of the embryos failed to completely germinate in all treatments evaluated (no results for this technique are given in Tables).

The percentages of germinated, rooted, and elongated embryos and the percentage of plants assessed under *in vitro* and pot conditions are shown in **Tables 1** and **2**, respectively. Significant differences between treatments were observed for all the variables evaluated in all cases. In general, there is a decrease in the percentage of rooted and elongated embryos yielding a low percentage of plants with

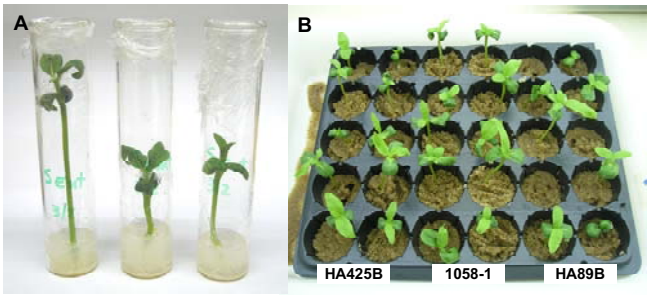


**Fig. 1 Sunflower plantlets developed from germination of immature embryos incubated for 15 days.** (**A**) *In vitro* technique. HA89B germinated plantlets are shown in test tubes. (**B**) Pot technique. Plantlets of the three inbred lines evaluated (HA425B, 1058-1 and HA89B) are depicted (two rows per inbred).

**Table 1** Germination and plant development from immature sunflower embryos under *in vitro* conditions. GE: percentage of germinated embryos, RE: percentage of rooted embryos, EE: percentage of elongated embryos and PL: percentage of plants.

| Genotype | Embryo manipulation | GE (%) | RE (%) | EE (%) | PL (%) |
|---|---|---|---|---|---|
| HA425B | Intact | 96 | 4 | 4 | 4 |
| | Cut | 79 | 0 | 0 | 0 |
| 1058-1 | Intact | 97 | 76 | 59 | 59 |
| | Cut | 100 | 97 | 93 | 80 |
| HA89B | Intact | 100 | 57 | 50 | 47 |
| | Cut | 100 | 97 | 55 | 45 |
| | $\chi^2$ | 21.41 | 109.79 | 73.66 | 58.58 |
| | p< | 0.001 | 0.001 | 0.001 | 0.001 |

**Table 2** Germination and plant development from immature sunflower embryos under pot conditions. GE: percentage of germinated embryos, RE: percentage of rooted embryos, EE: percentage of elongated embryos and PL: percentage of plants.

| Genotype | Embryo manipulation | GE (%) | RE (%) | EE (%) | PL (%) |
|---|---|---|---|---|---|
| HA425B | Intact | 100 | 33 | 30 | 27 |
| | Cut | 100 | 37 | 27 | 27 |
| 1058-1 | Intact | 100 | 80 | 73 | 60 |
| | Cut | 100 | 93 | 87 | 57 |
| HA89B | Intact | 97 | 57 | 53 | 17 |
| | Cut | 97 | 43 | 40 | 33 |
| | $\chi^2$ | 4.04 | 35.17 | 36.88 | 29.48 |
| | p< | 0.543 | 0.001 | 0.001 | 0.001 |

**Table 3** *G*-Test of independence for the factors genotype (G), embryo manipulation (M) and percentage of plants (PL) under *in vitro* and pot conditions.

| Hypothesis | D.F. | G-value | |
|---|---|---|---|
| | | *In vitro* | Pot |
| G*M independence | 2 | $0.15^{ns}$ | $0.00^{ns}$ |
| G*PL independence | 2 | $68.75^{**}$ | $29.52^{**}$ |
| M*PL independence | 1 | $0.35^{ns}$ | $0.23^{ns}$ |
| G*M*PL interaction | 2 | $4.41^{ns}$ | $0.42^{ns}$ |
| G*M*PL independence | 7 | $73.66^{**}$ | $30.17^{**}$ |

** p< 0.01; ns: non-significant

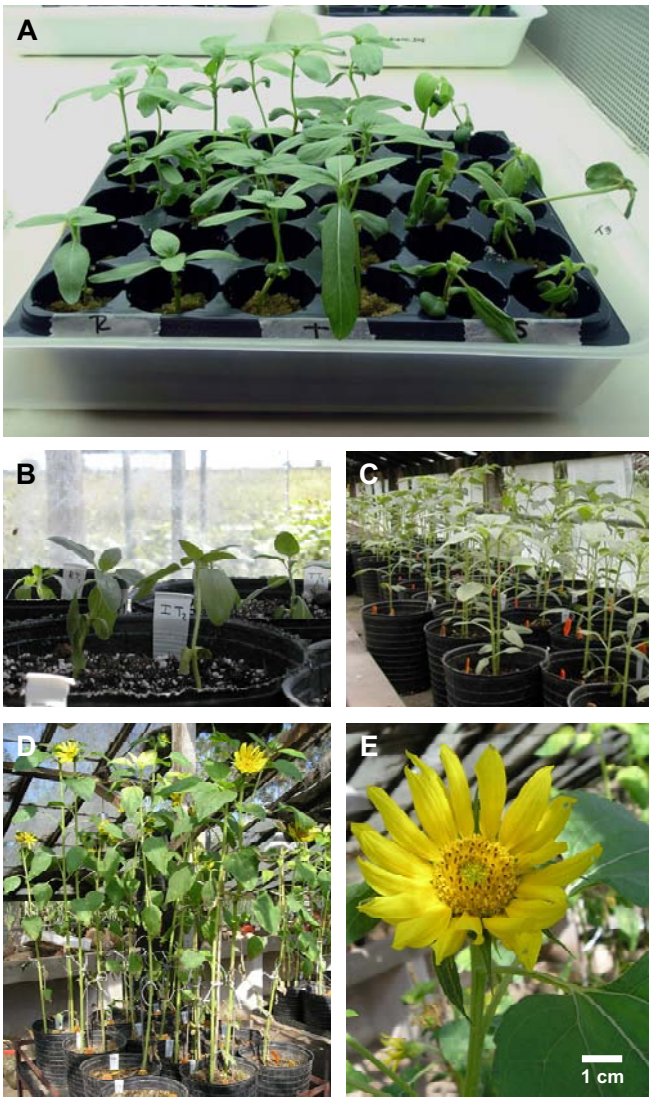


**Fig. 2 Screening for imazethapyr-resistant genotypes.** (**A**) Two-leaf stage plantlets from inbreds HA425B, 1058-1 and HA89B (imidazolinone resistant, intermediate and susceptible genotypes respectively) treated with nutritive solution plus 7.5 µM of herbicide (two rows per inbred). Susceptible plantlets showed arrested growth, internerval chlorosis, loss of turgency and finally became wilted. (**B-C**) Surviving plants growing in greenhouse 8 and 25 days after imazethapyr application, respectively. (**D-E**) Plants at their reproductive stage in the greenhouse.

regard to germinated embryos. Thus, germination was successful with both these techniques, but important losses occurred at each step of plantlet development (**Tables 1, 2**).

**Table 3** shows the test of independence (*G*-test) for genotype, embryo manipulation, and ability to develop plants. For both techniques, there is a significant lack of independence for genotype and ability to develop plants that mainly explains the dependency among genotype, embryo manipulation and ability to develop plants. Accordingly, genotypes differed in the total percentage of plants obtained. Inbred line 1058-1 showed the best *in vitro* growth performance (69%) followed by HA89B (46%), while HA425B was recalcitrant (2%). Under the non-sterile pot technique the inbred line 1058-1 also showed the best ability to develop plants from immature embryos (56%) while HA425B, and HA89B performed similarly (20%). As shown in **Table 3**, the ability to develop plants is not influenced by the embryo manipulation or by the interaction among genotype, embryo manipulation and ability to develop plants.

In general, the efficiencies of *in vitro* and pot germination techniques were very similar. The percentage of plants was 40% for the *in vitro* culture technique and 33% for pot germination technique ($\chi^2 = 1.93$, $p<0.165$).

Although the total percentages of plantlets did not differ significantly between the *in vitro* and pot germination technique, the latter proved to be simpler, less expensive and requires less skilled labour. For this reason, plantlets from the pot germination technique were used for screening herbicide resistance.

Five to six days after herbicide treatment, plantlets from the inbred line HA89B showed arrested growth, internerval chlorosis, loss of turgency and finally became wilted. These effects were more severe when exposed to 7.5 and 10 µM of imazethapyr. Conversely, plants of the lines HA425B and 1058-1 did not differ from the untreated control even when exposed to the highest dose of herbicide (**Fig. 2A**). At the stage when data was collected, intermediate resistant plants (from inbred 1058-1) could not be distinguished from the resistant ones by visual symptoms as they were not affected by the herbicide.

Surviving plants were transferred to greenhouse 8 days after imazethapyr application (**Fig. 2B**). Established plants were on average 72 cm tall and reached the reproductive stage 74 days after planting showing a 2-cm diameter head (**Fig. 2C-E**).

## DISCUSSION

Over the last years, *in vitro* techniques were increasingly used as a supplement to traditional breeding methods in the improvement of plants. In particular, embryo cultures have been used to overcome embryo inviability, seed dormancy and related problems as well as producing interspecific and intergeneric hybrids in several species (Thorpe 2007). In sunflower immature embryo culture is a popular technique used by sunflower breeding companies. Several authors studied the *in vitro* culture of immature sunflower embryos to rescue interspecific hybrids and speed up the vegetative cycle in a breeding program (Alibert *et al.* 1994).

Zorzoli *et al.* (1994) studied the effects of embryo age and culture media for *in vitro* germination of sunflower and determined that higher efficiencies were achieved for 13-15 day old embryos in free-hormone media. In general, embryos close to maturity do not require the addition of plant growth regulators (Hu and Ferreira 1998; Reed 2005).

Several authors previously described that media requirements depend on the stage of embryo development. Usually two phases of embryo development are identified: heterotrophic and autotrophic phases. The embryo in the heterotrophic phase requires a complex medium and high osmotic pressure. Conversely, during the autotrophic phase embryos can germinate on a simple inorganic medium supplemented with a carbon source (Bridgen 1994; Sharma *et al.* 1996).

Sunflower embryos developmental stages were studied by Espinasse *et al.* (1985). The *in vitro* development of embryos ranged in age from 2 to 14 days after anthesis was classified based on several parameters, including age, size and shape. The embryos cultured here were collected 15 days after pollination, presented uniform shape and size and were classified as fully developed cotyledons, stage described by Espinasse *et al.* (1985).

Plants obtained from immature embryos culture showed morphological modifications such as reduction of plant

height, head diameter and leaf size (Azpiroz *et al.* 1987; Zorzoli *et al.* 1994). Azpiroz *et al.* (1987) defined a relationship between the *in vitro* incubation time and the severity of these morphological changes. To avoid these difficulties Paul and Barthou (1994) studied the immature embryo culture under non-sterile conditions and proposed a simpler method that provided more vigorous plants. Torresán *et al.* (1996) also studied the dish germination technique with different embryo treatments and showed that seed manipulation was important (i.e. remove one-third of the blunt end). However, no differences were observed between intact or cut embryos in the techniques evaluated in this study (**Table 3**).

All embryos failed to germinate under dish germination technique conditions evaluated here. An important factor that might explain this failure is embryo orientation. Embryos were vertically oriented either in the *in vitro* or in the pot germination technique whereas in the dish technique embryos were horizontally placed. Pinto *et al.* (1994) studied the culture of immature peach embryos and found better results with support methods that oriented the embryos vertically. In addition, they observed an asymmetrical cotyledon growth from embryos cultured in horizontal position.

Embryos were lost in each step of plantlet development in the *in vitro* and pot germination technique evaluated. Almost all embryos effectively germinated but afterwards an average of 58% developed roots, 48% showed elongated hypocotyls and only 35% developed normal plantlets. These losses can be justified considering that immature embryos change their pattern of growth drastically in culture (Raghavan 1994).

Plants from the inbred line HA89B could be visually discriminated after herbicide application. This fact could allow the early elimination of susceptible plants when breeding for imidazolinone resistance and save space and resources. Immature embryos culture followed by selection of desired genotypes could be useful in assisting sunflower breeding programs. Pelletier *et al.* (1995) proposed a similar methodology to determine resistance to downy mildew (*Plasmopara halstedii*) in plants obtained from *in vitro* culture of immature embryos.

Plantlets produced by the pot technique were transferred to soil and concluded their cycle in greenhouse successfully. Besides, finishing the life cycle, another advantage of screening plants with the pot technique when compared to the *in vitro* technique is in the acclimatization period. Plantlets obtained from the pot assay were more vigorous, and adapted to the new environment in a shorter period, thus reducing the stress during acclimatization.

In the majority of breeding programs, the development of improved varieties involves growing large segregating populations, selection of desirable phenotypes and selfing those to develop inbred lines that subsequently will be used for hybrid production. Another breeding scheme is the conversion of elite inbred lines by backcrossing. Both processes are time and space consuming. The pot germination technique described in this work could be useful to assist these breeding schemes. This simple and reproducible technique that requires low inputs offers an opportunity to improve selection efficiency by shortening breeding cycle and early screening resistant genotypes.

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