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Development of transgenic imazapyr-tolerant cowpea (*Vigna unguiculata*)

C. T. Citadin · A. R. R. Cruz · F. J. L. Aragão

Abstract

Key message **Here we present the development of cowpea lines tolerant to a herbicide from imidazoline class (imazapyr). Plants presented tolerance to fourfold the commercial recommended dose for weed control.**

Abstract Cowpea is one of the most important and widely cultivated legumes in many parts of the world. Its cultivation is drastically affected by weeds, causing damages during growth and development of plants, competing for light, nutrients and water. Consequently, weed control is critical, especially using no-tillage farming systems. In tropical regions, no-till farming is much easier with the use of herbicides to control weeds. This study was conducted to evaluate the possibility of obtaining transgenic cowpea plants resistant to imidazolinone, which would facilitate weed control during the summer season. The biolistic process was used to insert a mutated acetohydroxyacid synthase coding gene (*Atahas*) which confers tolerance to imazapyr. The transgene integration was confirmed by Southern blot analysis. Out of ten lines tested for tolerance to 100 g ha^{-1} imazapyr, eight presented some tolerance. One line (named 59) revealed high herbicide tolerance and developmental growth comparable to non-transgenic plants. This line was further tested for tolerance to higher herbicide concentrations and presented tolerance to 400 g ha^{-1} imazapyr (fourfold the commercial recommended dose) with no visible symptoms. Line 59 will be the foundation for generating imidazolinone-tolerant cowpea varieties, which will facilitate cultivation of this crop in large areas.

Keywords Genetic engineering · Herbicide tolerance · Imidazolinone · Plant transformation

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Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is one of the most important and widely cultivated legumes in many parts of the world, particularly in Africa, Europe, Latin America and Asia. Approximately 4 million tons of dry cowpea grains are produced annually in an area covering about 10 million hectares (for a review see Citadin et al. 2011). Besides being a very important source of carbohydrates, lipids, minerals and vitamins, including folate, thiamin and riboflavin (Nielson et al. 1993), the plant is distinguished by its adaptability to poor soil and can be grown in semi-arid regions with little technology employed (Singh et al. 2003).

Cowpea cultivation is drastically affected by weeds, causing damages during growth and development of plants, competing for light, nutrients and water. If weeds are not correctly controlled, the yield losses could be up to 90 % (Freitas et al. 2009). Some species of weeds like *Sida rhombifolia*, *Waltheria indica*, *Cleome affinis* and *Herissantia crispa* could also serve as alternative hosts for pathogens that cause diseases in cowpea, increasing losses (Assunção et al. 2006). In addition, hemi parasitism caused by *Striga gesnerioides* and *Alectra vogelii* is a serious problem for cowpea cultivation in Africa, affecting roots and plant development (Li et al. 2009).

Imidazolinone tolerance was achieved by both conventional and molecular breeding in rice (Tan et al. 2005),

maize (Newhouse et al. 1991), oilseed rape (Swanson et al. 1989), soybean (Aragão et al. 2000), sugarcane (Stuart et al. 2010), sugar beet (Kishchenko et al. 2011) and cotton (Rajasekaran et al. 1996). The mechanism of action of imidazolinone herbicides, such as imazapyr, is the inhibition of the enzymatic activity of acetohydroxyacid synthase (AHAS; acetolactate synthase, acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18), which catalyzes the initial step in the biosynthesis of isoleucine, leucine and valine (Shaner et al. 1984).

Cowpea cultivation areas are on the increase in some areas of developing countries, such as Midwestern Brazil, where an area of about 150,000 hectares is being cultivated. With such large areas being cultivated, there is interest in using no-tillage farming systems, which are attracting increased attention worldwide. No-tillage farming provides several environmental benefits for soil structure and aquatic resources, as well as providing positive effects through the storage of organic carbon, which, as carbon dioxide, acts in the atmosphere as a greenhouse gas. Herbicides serve as useful tool for weed management, particularly in the first years after shifting from conventional farming to no-till farming. In tropical regions, it is much easier to manage no-till farming with the use of herbicides (Friedrich 2005). In this context, a herbicide-tolerant cowpea would be useful for cultivation in a no-tillage agricultural system. In this work the achievement of transgenic cowpea lines with high imazapyr tolerance is reported, involving the introduction of a mutant *ahas* gene isolated from *Arabidopsis thaliana* (*Atahas*) into the cowpea genome.

Materials and methods

Cowpea transformation

Transformation was carried out with the vector pAC321 (Aragão et al. 2000; Rech et al. 2008) as previously described (Ivo et al. 2008). The pAC321 vector was digested with *Fsp*I prior to transformation to excise the *bla* gene, which confers bacterial tolerance to β-lactams (ampicillin). Basically, mature seeds of cowpea (cv. Nova Era) were surface sterilized and soaked in distilled water for 16–18 h. Then the embryonic axes were excised from the seeds and the apical meristems were exposed by removing their primary and primordial leaves. The embryonic axes were placed with the apical region directed upward in 5 cm Petri dishes containing MS medium, immediately before the bombardment. The bombardment was conducted using a high-pressure helium-driven particle acceleration device built in our laboratory. The embryonic axes were cultivated on MS medium containing 5 mg L^{-1}

BAP to induce multiple shoot development and 300 nM imazapyr. The PCR-positive shoots were rooted, acclimatized and allowed to set pods.

Screening of transgenic plants by PCR

The analysis of the transgenic lines T_0 and T_1 generation was carried out by amplifying a fragment of the *Atahas* gene by PCR. DNA isolation and PCR were carried out using the Extract-N-Amp Plant PCR Kit (Sigma) according to the manufacturer's protocol. The primers AHASP (5′-ACTAGAGATTCCAGCGTCAC-3′, within the *Atahas* promoter) and AHAS500C (5′-GTGGCTATACAGATA CCTGG-3′, within the *Atahas* coding sequence) were used to amplify a 685-bp sequence. DNA was denatured for 5 min at 95 °C in the MyCycler thermal cycler (BioRad) and amplified for 35 cycles (95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) with a final cycle of 7 min at 72 °C. The reaction mixture was then loaded onto 1 % agarose gel and visualized under UV light following ethidium bromide staining.

Southern blot analysis

Genomic DNA was isolated from 3 g of leaves using the CTAB method described by Doyle and Doyle 1987. Southern blotting was carried out as described by Sambrook and Russell (2001). Genomic DNA (30 μg) was digested with *Xba*I, separated on 0.8 % agarose gel and transferred to a nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech). Hybridization was carried out using the probe comprising a region at 1,701–2,363 pb from the *Atahas* promoter gene labeled with $^{\alpha}$32P dCTP using a random primer DNA labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The bands were visualized with a fluorescent image analyzer (FLA-3000) (FUJIFILM).

Progeny analysis

The analysis of the T_1 generation transformants (self-pollinated plants) was carried out by amplifying a fragment of the *ahas* gene by PCR as described. Chi-square (χ^2) analyses were performed to determine if the observed segregation ratio was consistent with a Mendelian ratio of 3:1 (for one locus) or 15:1 (for two loci). Where appropriated, Yates Chi-squared test was used.

Test for tolerance to imazapyr

Ten seeds collected from T_3 generation of transgenic lines were sown in 5 dm^3 plastic pots containing autoclaved fertilized soil. Once the plants produced the first trifoliate

leaves, they were sprayed with the herbicide (imazapyr) at a concentration of 100 g ha^{-1}, observed and photographed after 3 weeks. Additionally, concentrations of 100, 200, 300 and 400 g ha^{-1} were used to determine the tolerance in some transgenic lines.

Measurement of AHAS activity

AHAS activity was analyzed by the colorimetric enzymatic described by Sato and Takamizo (2009) except that the bispyribac-sodium (BS) was replaced by 0.3 µM imazapyr.

Results

The apical regions of cowpea embryonic axes were bombarded with the plasmid pAC321, which contains a mutated *Atahas* gene from *A. thaliana* that confers tolerance to imidazolinone. Following bombardment the embryonic axes were cultured in a selection and multiple shooting inductions medium. As soon as the plantlets developed vigorous roots they were tested by PCR (Fig. 1). Ten PCR-positive plantlets were acclimatized and transferred to soil. After developing into mature plants, it was observed that all plants presented a normal phenotype, were fertile and set pods and seeds. These plants were grown to produce seeds and we advanced to T_3 generation. Southern blot analyses with the T_3 generation of transgenic cowpea lines were conducted to evaluate the integration of the introduced *Atahas* gene. The results showed the presence of the *Atahas* elements in all transgenic lines (Fig. 2).

The progeny of the ten self-fertilized transgenic lines was screened by PCR analysis for the presence of the *ahas* gene. All lines transferred the foreign genes in a Mendelian fashion. Chi-square (χ^2) analysis revealed that the lines 52 and 99 presented a segregation ratio of 15:1 and the lines 12, 21, 39, 59, 71, 110, 112 and 119 presented a segregation ratio of 3:1 (Table 1).

Since the vector pAC321 has two sites for the restriction enzyme XbaI flanking the *Atahas* cassette, Southern analyses allowed us to confirm that the *Atahas* cassette was integrated into the cowpea genome. Except for line 52, all lines presented the expected 5.8 kb fragment, suggesting
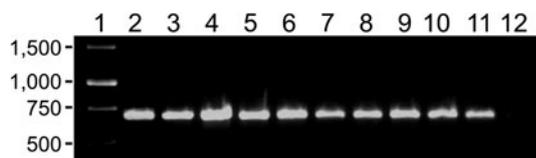

Fig. 1 PCR analysis of transformed cowpea plants with the plasmid pAC321. *Lane 1* standard molecular size (GeneRuler 1 kb DNA Ladder, Fermentas; in bp). *Lanes 2–11* transformed lines, *Lane 12* non-transformed plant
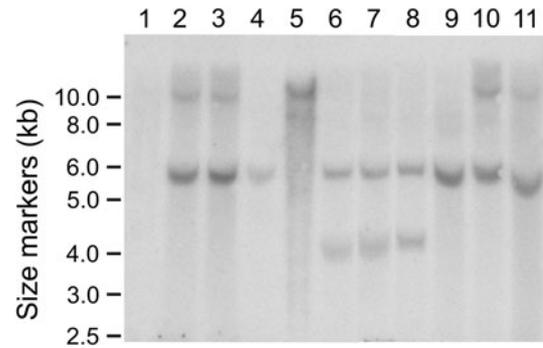

Fig. 2 Southern blot analysis of putative transformed lines. Genomic DNAs were digested with XbaI, transferred to a nylon membrane and hybridized with probe against *Atahas* promoter region as described above. *Lanes 2–10* Independently transformed lines (line 12, 21, 39, 52, 59, 71, 99, 110, 112 and 119). *Lane 1* non-transformed plant. Molecular size markers are indicated on the *left*

Table 1 Segregation analysis of self-fertilized transgenic cowpea plants in the T_1 generation

Lines	Positive[a]	Negative[a]	Segregation ratio	χ^2	P[b]
12	22	5	3:1	0.60	0.44
21	36	8	3:1	1.09	0.30
39	56	22	3:1	0.42	0.51
52	38	2	3:1	9.10	0.04
			15:1	0.40	0.74
59	12	5	3:1	0.17	0.68
71	46	12	3:1	0.57	0.45
99	82	5	3:1	17.19	0.00
			15:1	0.03	0.85
110	24	12	3:1	1.33	0.25
112	35	12	3:1	0.01	0.93
119	45	13	3:1	0.21	0.65

[a] Data are based on PCR analysis

[b] P is the probability that the observed ratios reflect the expected segregation ratio of 3:1 or 15:1

that the *Atahas* cassette was completely integrated (Fig. 2). DNA isolated from non-transformed plants did not hybridize with the *Atahas* probe (Fig. 2, lane 1). Lines 12, 21, 112 and 119 presented an additional fragment higher than 5.8 kb. Lines 59, 71 and 99 presented an additional fragment lower than 5.8 kb.

All transgenic lines were treated with imazapyr herbicide at a concentration of 100 g ha^{-1}. Lines showed different levels of tolerance to imazapyr after 2 weeks (Fig. 3a). Red vein symptoms typically seen in imidazolinone-treated plants were observed in almost all lines, except in line 59 (Fig. 3b). No tolerance was observed in line 52 and non-transgenic plants, which died 2 weeks after herbicide treatment. Lines 99, 112, 119, 110, 21, 71, 39 and 12 presented mild symptoms, produced seeds and
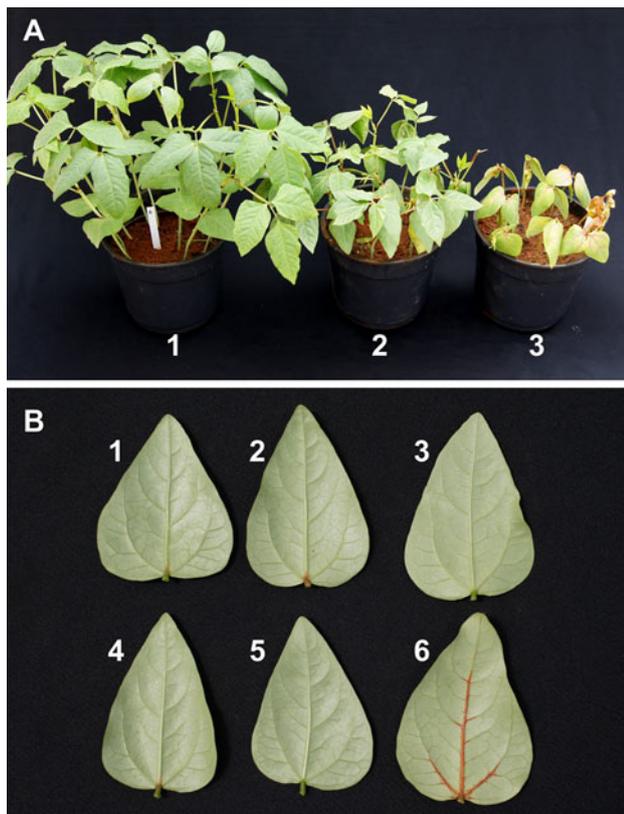
Fig. 3 Test of progeny of transgenic cowpea lines for tolerance to the herbicide imazapyr. a Transgenic lines 59 (1), 12 (2) and non-transgenic (3) 2 weeks after herbicide treatment (100 g ha^{-1} imazapyr). b Details of the abaxial leaves of the transgenic lines 99 (1), 112 (2), 110 (3), 119 (4), 59 (5) and non-transgenic (6), removed from plants treated with 100 g ha^{-1} imazapyr

presented multiple branches. Line 59, which showed high tolerance to herbicide and developed normally with no observed symptoms was further tested for tolerance to 100, 200, 300 and 400 g/ha imazapyr (Fig. 4). Results showed that this line did not present any observed herbicide symptoms at these doses 20 days after treatment (Fig. 4). Line 59 produced a similar number of seeds when compared with non-transgenic plants.

AHAS activity in line 59 (which presented high tolerance to imazapyr) and line 99 (which presented mild symptoms when treated with the herbicide) was analyzed by colorimetric enzymatic assay. This assay estimates AHAS activity in plant tissues with or without herbicide treatment based on a comparison of acetoin accumulation (Gerwick et al. 1993). Assay carried out with leaf tissues from both control and transgenic plants produced pink coloration under incubation without imazapyr (Fig. 5a). Transgenic leaves that were incubated with imazapyr produced pink coloration, while control plants produced brown/light pink coloration (Fig. 5a). AHAS activity in leaves that were treated with imazapyr was measured and

revealed higher enzymatic activity in the herbicide-tolerant plants (1.76 for the line 59 and 0.99 for the line 99) than in the control (0.43). Control leaves without imazapyr revealed equivalent enzymatic activity (1.21) to transgenic leaves treated with the herbicide. In addition, line 59 presented the higher AHAS activity in leaves treated and not treated with the herbicide (Fig. 5b).

Discussion

We reported here the achievement of transgenic cowpea tolerant to herbicidal molecule imazapyr (imidazolinone) using a mutated *Atahas* gene from *A. thaliana*. Among the ten genetically modified lines analyzed by Southern blot, the cassette for the *Atahas* gene expression was present. Four lines (12, 21, 112, 119) presented the expected 5.8 kb band, corresponding to the *Atahas* expression cassette and an additional fragment with size higher than 10 kb band. Three lines (59, 71 and 99) presented an addition fragment ca. 4 kb. This suggests that these lines could have partial insertions of the *Atahas* cassette. Although the occurrence of primary chimeric transgenic plants has been commonly observed in leguminous plants transformed by particle bombardment of meristematic tissues (Aragão et al. 1996; Christou et al. 2006), no chimeric cowpea plants were detected since all lines presented a Mendelian segregation ratio in the first generation.

Plants of the ten genetically modified lines were tested for tolerance to 100 g ha^{-1} imazapyr. Nine lines presented herbicide tolerance, ranging from mild symptoms (lines 99, 112, 119, 110, 21, 71, 39 and 12) to symptomless plants (line 59). This distinction probably reflects the integration of the transgenes in different genome positions. Moreover, the expression of a transgene located in active chromatin or heterochromatin is highly variable, even among lines independently transformed with the same construct. Many factors may be responsible for variable transgene expression, including the tendency for exogenous DNA to undergo inverted repeat rearrangement prior to transformation by using direct methods, effects related to integration position and DNA hypermethylation (Kohli et al. 2003).

Colorimetric enzymatic assay revealed that AHAS activity was higher in the line 59, which presented higher tolerance to imazapyr. This result corroborated the phenotype observed in plants sprayed with imazapyr. Higher enzyme activity observed in line 59 could be explained by a synergism generated by the presence of both endogenous and foreigner AHAS proteins.

Line 52 did not present tolerance to 100 g ha^{-1} imazapyr. This line did not show the expected 5.8 kb fragment corresponding to the *Atahas* gene in the Southern
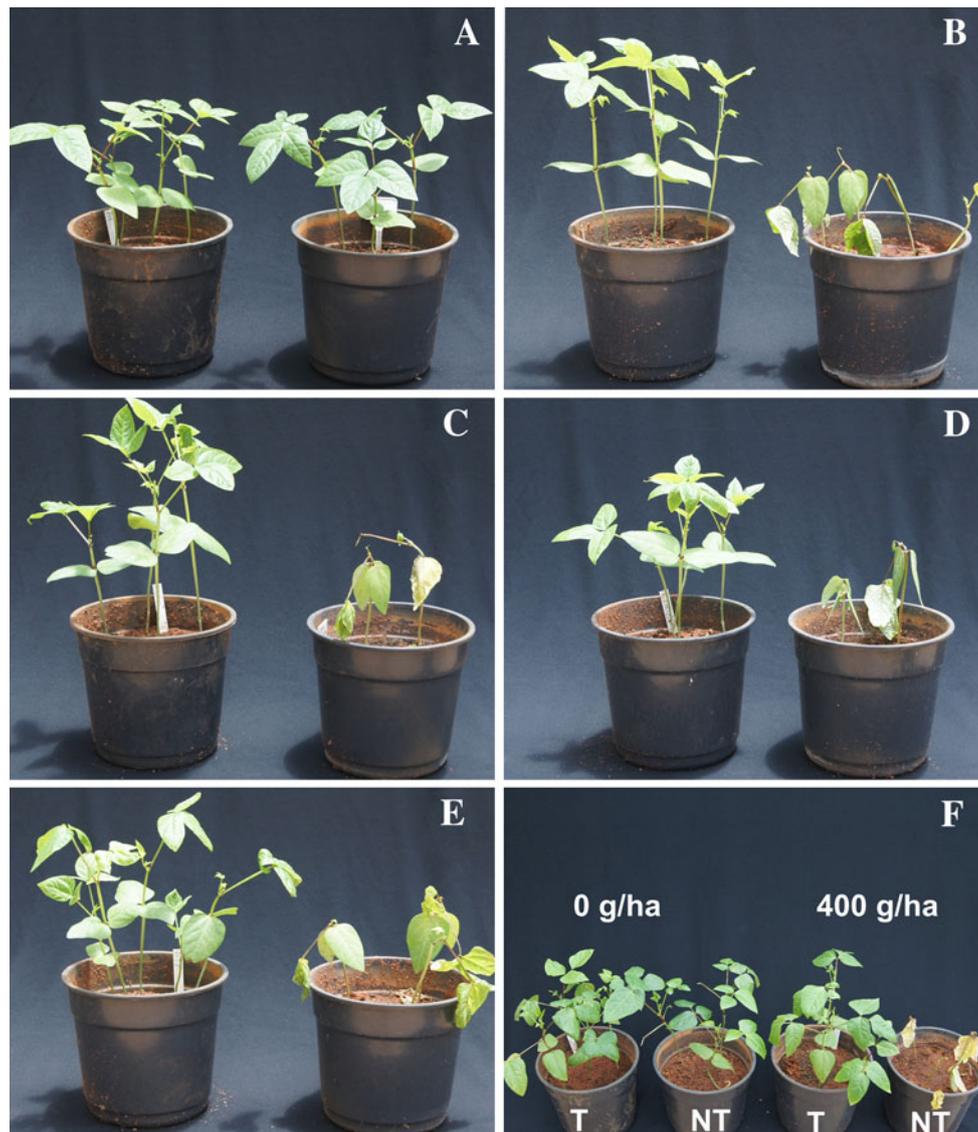
Fig. 4 Evaluation of transgenic line 59 for tolerance to four concentrations of the herbicide imazapyr. Non-sprayed plant (**a**), and plants sprayed with 100 g ha^{-1} (**b**), 200 g ha^{-1} (**c**), 300 g ha^{-1} (**d**) and 400 g ha^{-1} (**e**) imazapyr (11 days after treatment). **f** Plants after 20 days of herbicide treatment. From **a–f**, plants on the *left* are transgenic (line 59) and plants on the *right* are non-transgenic (control)

analysis, suggesting the absence of a functional copy of the gene. The lack of sufficient expression of the mutated *Atahas* gene in line 52 would be an explanation for its low tolerance to imazapyr.

Questions regarding biosafety issues have been raised because of the presence of genes for antibiotic resistance in transgenic plants (Dale 1999, Tuteja et al. 2012), and sequences coding for resistance to some antibiotics are consequently being avoided for the development of commercial cultivars. Use of linear vectors is important to eliminate undesirable antibiotic selective genes. However, for dry bean, the transformation efficiency decreased when a linear vector was used (Vianna et al. 2004). We decided to use a linearized vector in which the gene for tolerance to ampicillin was inactivated. The ability to genetically engineer cowpea is still not trivial (Citadin et al. 2011; Diouf 2011) and the previously reported efficiency of transformation using circular vectors and the biolistic process was 0.9 % (Ivo et al. 2008). In contrast to other reports, the frequency of transformation remained similar when compared with the transformation system using circular vectors.

There is a considerable interest in generating herbicide-tolerant cowpea plants. Imidazolinone tolerance was achieved by both conventional and molecular breeding in rice, soybean, sugar beet and sugarcane (Aragão et al.
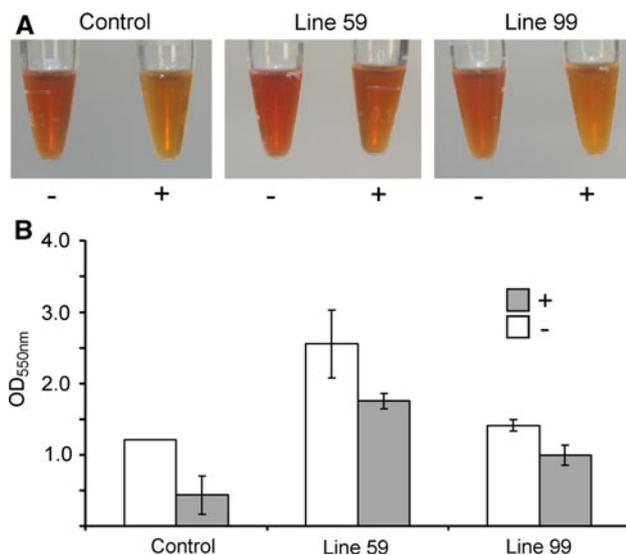
Fig. 5 Colorimetric enzymatic assay to determine AHAS activity in leaves of control (non-transgenic plants) and transgenic plants. The leaf tissues were incubated with (+) or without (−) imazapyr. **a** Comparison of acetoin accumulation in leaves from control and transgenic lines 59 and 99. **b** Measurement of AHAS activity. *Error bars* represent the SE ($n = 3$)

2000; Tan et al. 2005; Stuart et al. 2010; Vianna et al. 2011, Kishchenko et al. 2011). Line 59 showed high tolerance to up to 400 g ha^{-1} imazapyr, which is fourfold the commercial recommended dose for weed control in a number of crops. Additionally, this line presented a Mendelian segregation (3:1) in the F$_1$ generation. It makes the transgene easier to transfer to other genotypes. This event behaved similarly to a non-transgenic event under greenhouse conditions and has been included in Embrapa's breeding program. Line 59 has the potential to be the foundation for new cowpea varieties tolerant to imidazolinones, which will facilitate cultivation of this crop in large areas of tropical regions.

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