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ARTICLE

Behavior of soybean genotypes transformed with the *AHAS* gene which confers tolerance to the herbicide imazapyr

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ABSTRACT *- The transformation of the soybean cultivars BR-16 and Doko with the* AHAS *gene resulted in the development of imazapyr-tolerant herbicide genotypes. A winter greenhouse trial was conducted in order to compare the conventional genotypes BR-16 and Doko with their transformed counterparts under imazapyr application. Some aspects (number of pods and seeds weight) were evaluated and observations showed that genotype BR-16-AHAS was more affected than Doko-AHAS compared to their conventional counterparts. Another experiment was conducted with the two transformed genotypes (AHAS) at a dosage of zero and 100 g i.a. ha^{-1} imazapyr application in the summer. The relative responses of the two genotypes were similar to the winter results but the herbicide effects differed regarding mainly the most affected position in each genotype. The herbicide imazapyr applied (100 g i.a. ha^{-1}) onto AHAS gene-transformed plants may induce the development of sterile pods. The herbicide and not the inserted gene is responsible for this effect.*

Key words: *Arabidopsis thaliana*, genetically modified organisms-GMO, teratogenic effect, phytotoxicity, genotype x environment interaction.

INTRODUCTION

Genetic engineering has made the generation of non-existing genetic combinations in nature possible and mainly the gene transference between reproductively isolated species, creating the transgenics, or genetic modified organisms (GMO). Genes of any living organism can be accessed, isolated, modified for a better expression in specific tissues, and inserted into the genome of the most cultivated species (Gasser and Fraley 1989).

In soybean, one of the first applications of genetic engineering was the development of glyphosate herbicide tolerance (Padgette et al. 1995), a technology that achieved great commercial success in the USA and mainly in Argentina. Aragão et al. (2000) obtained imazapyr-tolerant soybean plants through the biobalistic transformation technique, where the mutant gene *AHAS*, isolated from *Arabidopsis thaliana*, was inserted.

Imazapyr is an herbicide with a large spectrum of action; it controls most annual and perennial monocotyledonous as well as dicotyledonous species (Beardmore 1991). This herbicide belongs to the chemical group of imidazolinones and inhibits the acetolactate synthase enzyme (ALS) which participates in the metabolic pathway of the biosynthesis of three essential amino acids (valine, leucine, and isoleucine), and has the ability to cause plant death. The ALS inhibitor

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herbicide is used in large scale owing to its low toxicity to animals and its high efficiency at low doses (Vidal 1997).

Aragão et al. (2000) studied four events in soybean plants expressing the *AHAS* gene, introduced by biobalistics: three in the BR-16 cultivar, adapted to the Southern part of Brazil, and one in the Doko cultivar, adapted to Central Brazil. These authors observed that, at the initial development stages of soybean plants, the BR-16 events presented a slightly superior tolerance to the herbicide in comparison to Doko. However, at mild temperatures, the pollen development is apparently affected in BR-16 but not in Doko. The best event in BR-16 and the event in Doko were selected for their superior phenotypical performance to participate in this study.

Objectives of this study were to compare the performance of conventional genotypes untreated with imazapyr herbicide to AHAS-treated genotypes and to evaluate the effect of imazapyr treatment on the BR-16-AHAS and Doko-AHAS events.

MATERIAL AND METHODS

The BR-16-AHAS and Doko-AHAS genotypes used in this study are events developed in soybean cultivars through biobalistics at Embrapa Genetic Resources and Biotechnology and passed on to Embrapa Soybean in 1999. BR-16 and Doko are the conventional soybean cultivars used to obtain the events. The first part of the study was carried out in the winter and the second in the summer of 2001/2002.

All experiments were carried out in a greenhouse, under strict observation of biosafety rules. The plants were cultivated in 23 cm diameter, 8 liter plastic pots. The substrate consisted of a mixture of 60% fertilized subsoil, 20% sand, and 20% organic compost. Fertilizer was applied according to the substrate analysis. Nitrogen was not used and *Bradyrhizobium japonicum* was always inoculated. The seeds were placed in a germinator and transplanted on the fourth day. Imazapyr was usually applied 13 to 15 days after transplant. Some plants of the non-transformed genotypes were always treated to control the effectiveness of the application.

Winter experiments

Two experiments, one for the BR-16 cultivar and another for the Doko cultivar, were set up in July 2001 in a greenhouse. They were sown on 02/07/2001 and transplanted on 06/07/2001. Artificial light was supplied at night until 26/07/2001 for better plant development under winter conditions. Temperature and relative air humidity were recorded using a thermohygrograph. The treatments were imazapyr-untreated conventional soybean and AHAS-treated soybean. A randomized complete block design was used with seven replications. Plots consisted of one pot containing two plants, of which the plant means were used. The imazapyr herbicide was applied on 19/07/2001 at 100g i.a. ha^{-1}. At maturity, the plants were divided into thirds and the number of pods (Np) and seeds (Ns) were counted and the grains weighed (W) for each plant part (upper, mid, and lower). The mean number of seeds per plot (Nsp) and the weight of 100 seeds (W100) were calculated for each plot. Analyses of variance were performed for each cultivar, according to the statistical model $Y_{ijk} = m + b_j + t_i + p_k + tp_{ik} + e_{ijk}$, where Y_{ijk} corresponds to observations made on the k position of the treatment i in block j, m is the mean trait effect, b_j the block effect, t_i the treatment effect, p_k the effect of position, tp_{ik} the interaction between treatment i for position k, and e_{ijk} is the residual effect of each plot.

Summer experiment

An experiment including the events BR-16-AHAS and Doko-AHAS was set up in a greenhouse in December 2001 (sown 01/12/2001 and transplanted 05/12/01). The two events were submitted to 0 and 100g i.a. ha^{-1} imazapyr according to a randomized complete block design with 13 replications. The experimental plot consisted of one pot containing two plants of which the plant means were considered. The herbicide was applied 13 days after transplant when the pots were separated for application and later replaced in their original positions. The traits, assessed in the different thirds of the plants were the same as in the winter experiment. Additionally, the plant height and number of nodes (Nno) were assessed in this experiment. The analysis of variance was carried out according to the following statistical model:

$$Y_{ijkl}=m+g_i+t_j+g/t_{ij}+p_k+gp_{ik}+tp_{jk}+gtp_{ijk}+e_{ijkl} \quad ,$$

where Y_{ijk} is the observation of genotype i with treatment j in position k in the replication l; m is the mean effect for the trait; g_i is the genotype i effect; t_j is the treatment effect j; g/t_{ij} is the interaction between genotype i and treatment j; p_k is the effect of position k; gp_{ik} is the interaction between genotype i for position k; tp_{jk} is the interaction of treatment j by position k; gtp_{ijk} is the triple interaction of genotype i by treatment j by position k; and e_{ijk} is the residual effect of each plot.

RESULTS AND DISCUSSION

Winter experiments

The treatments with imazapyr herbicide were effective at interrupting growth immediately with subsequent death of the apical meristem, leaves, and stems of the non-tolerant patterns. In the greenhouse under artificial irrigation these plants normally emit secondary branches from the lower part, flower again, and produce pods with viable seeds.

All plants of both the Doko and the BR-16 cultivars presented a development in height and phenotypic development that was compatible with the characteristics of each cultivar. The BR-16 cultivar responded satisfactorily to light treatment that permitted good plant development even in wintertime. The temperature inside the greenhouse was always kept between 18 and 27 ºC and relative humidity ranged from 35 to 90%. The coefficients of variation (CV%) of the analyses of variance ranged from 11.5% for Nsp to 36% for W, values considered normal for these traits when assessed on individual plants (Table 1).

The analysis of variance (Table 1) showed that there was significance ($P < 0.05$) for BR-16 in treatment and position for all characteristics and the T x P interaction for all except for Nsp. For the Doko cultivar, there was significance for the position for all traits and treatment for W100. This analysis showed that the various traits assessed in BR-16 and Doko had a different expression in the different thirds of the plants and that these relative differences were affected by treatment (T x P interaction) only in BR-16 (Np, Ns, W, and W100). For Doko, the difference between the untreated conventional cultivar and the imazapyr-treated AHAS-cultivar was only detected for W100.

It was observed that all traits were affected by the treatment in BR-16 when the effects of treatments on the whole plants were considered (general, Table 2). The BR-16-AHAS presented a significant decrease in Np, Ns, W, and Nsp, and increase in W100. In Doko the traits were not affected except for W100, which increased significantly with the use of the technology. Because of the significance of the T x P interaction for BR-16, these effects were partitioned by position to ascertain the treatment effects. In the upper plant parts, there was a significant difference for most of the traits except Nsp in BR-16 and weight and Nsp in Doko. In the mid part, all traits in BR-16 were affected by the treatment, whereas in Doko most traits were not affected, except for W100. In the lower part the treatment affected only Nsp and W100 traits in BR-16.

By the analysis of the AHAS technology (gene + post-emergence herbicide application) in this experiment, it was clear that the BR-16-AHAS event was much more affected than the Doko-AHAS event when compared to the respective conventional cultivars, confirming observations made by Aragão et al. (2000). BR-16 was affected in all plant parts, especially the upper and mid thirds, while Doko was affected mainly in the upper third and compensated these losses in the lower third of the plant, balancing the result for the whole plant. Because the effects of the *AHAS* gene and the imazapyr herbicide were confounded, it was not possible to indicate the cause of these effects in this experiment. The summer experiment was conducted for further investigation.

Table 1. Mean squares for number of pods (Np), number of seeds (Ns), grain weight (W in g), number of seeds per pod (Nsp), and weight of one hundred seeds (W100 in g) assessed in conventional and AHAS genotypes derived from the BR-16 and Doko soybean cultivars, sown in July 2002 in a greenhouse

Sources of Variation	df	Np	Ns	W	Nsp	W100
			BR-16			
Block	6	13.5	35.1	1.09	0.01	3.3
Treatments (T)	1	672.0 **	3520.0 **	56.9 **	1.23 **	345.8 **
Position (P)	2	671.1 **	1442.9 **	46.1 **	0.23 *	29.9 *
T x P	2	65.6 *	283.3 **	14.4 **	0.09	21.8 *
Error	30	20.4	54.5	2.7	0.04	6.7
Mean		13.9	24.2	4.6	1.7	20.5
C.V. (%)		32.5	30.5	35.9	12.2	12.6
			Doko			
Block	6	8.5	27.8	1.6	0.07	8.1
Treatments (T)	1	14.3	64.4	3.8	0.003	147.9 **
Position (P)	2	277.4 **	1438.6 **	54.6 **	0.59 **	14.7 *
T x P	2	38.5	214.8	3.8	0.04	7.7
Error	30	16.8	79.4	3.2	0.06	4.1
Mean		15.0	31.0	5.8	2.1	18.9
CV (%)		27.3	28.8	30.5	11.5	10.7

**; * Significant at the levels 1% and 5% of probability

Table 2. Means of the number of pods (Np), number of seeds (Ns), grain weight (W in g), number of seeds per pod (Nsp), and weight of one hundred seeds (W100 in g) obtained in the upper, mid and lower third of the plants and from the whole plant (general) in conventional and AHAS-treated genotypes derived from the BR-16 and Doko soybean cultivars sown in July 2002 in a greenhouse

	BR-16					Doko				
Treatments	**Np**	**Ns**	**W**	**Nsp**	**W100**	**Np**	**Ns**	**W**	**Nsp**	**W100**
					Upper					
Conventional	10.86 a[1]	21.57 a	4.15 a	1.98 a	19.66 b	13.50 a	29.93 a	5.09 a	2.27 a	17.16 b
AHAS-treated	3.57 b	6.00 b	1.39 b	1.72 a	23.34 a	10.07 b	22.00 b	4.84 a	2.17 a	22.47 a
					Mid					
Conventional	19.71 a	38.86 a	6.64 a	1.98 a	17.02 b	21.43 a	45.64 a	7.93 a	2.18 a	17.27 b
AHAS-treated	7.07 b	10.50 b	2.54 b	1.45 b	25.84 a	18.71 a	39.64 a	8.22 a	2.12 a	20.88 a
					Lower					
Conventional	23.07 a	39.57 a	6.46 a	1.72 a	16.35 b	11.79 a	21.07 a	3.54 a	1.78 a	16.62 a
AHAS-treated	19.00 a	28.57 a	6.34 a	1.48 b	21.37 a	14.43 a	27.57 a	5.29 a	1.88 a	18.96 a
					General					
Conventional	17.88 a	33.33 a	5.75 a	1.89 a	17.68 b	15.57 a	32.21 a	5.52 a	2.08 a	17.02 b
AHAS-treated	9.88 b	15.02 b	3.42 b	1.44 b	23.41 a	14.40 a	29.74 a	6.12 a	2.02 a	20.86 a

[1]Treatment means in the same column followed by the same letter do not differ in the F test at 5% probability

Summer experiment

In the summer, Doko and BR-16 plants presented good development and plant height without needing artificial light. The temperatures ranged from 20 to 30 °C and the relative humidity from 40 to 90%. As in the winter, the imazapyr treatment was effective in the summer experiment, causing apical meristem death in all non-transformed controls. The coefficients of variation (CV%) ranged from 4% for Nno to 33% for W.

The analysis of variance (Table 3) revealed significant difference among the genotypes for all characteristics, among treatments for almost all except Ns and weight, and the G x T interaction for Nsp, W100, height, and Nno. In this first part of the analysis, where the whole plant was considered without taking the position effect into account, it was observed that imazapyr herbicide application significantly ($P<0.05$) affected the number of pods on the plants but did not influence the number of seeds or the grain weight. As the treatment was homogeneous for both cultivars, it was concluded that the treatment effects were homogeneous for the two genotypes when the whole plant was considered. This was not the case for the traits Nsp, W100, plant height, and Nno where a differentiated response of the genotypes to the imazapyr application was detected. The treatment with imazapyr caused a reduction in plant height and consequently in the number of nodes and the reduction was proportionally greater for the Doko genotype (Table 5).

Table 3 further shows that there was significance in the effects of position and the G x P interaction for all traits and for the T x P interaction in the traits Np, Ns, W and Nsp for the triple interaction (G x T x P) in the traits Np, Ns and W. Table 4 shows the comparison of treatment means (F test) carried out for each G x P combination, derived from the analyses of variance. When the effects of each plant third were considered separately given by the position effect, it was proved that the traits generally have a differential expression according to their position on the plant. This differential expression usually depended on the genotype assessed. This had been expected, because the Doko cultivar tends to produce proportionally more pods on the mid and upper plant parts compared to the BR-16 cultivar (Table 4). This differential expression was also affected by imazapyr application (T x P interaction) which resulted in a greater proportion of pods in the lower third of the plants, although in Doko it was not significant. The significance of the triple interaction (G x T x P), although normally difficult to interpret, can be verified in Table 4, as the last effect discussed (greater Np on the lower plant parts) occurred with greater intensity in the BR-16 cultivar compared to Doko. This effect can be verified by observing the significances obtained in the upper and lower thirds of the BR-16 plants that did not occur in Doko.

In the summer experiment, the means for Np, Ns, weight, and Nsp were much higher than the means of the winter

Table 3. Mean squares for number of pods (Np), number of seeds (Ns), number of seeds per pod (Nsp), and weight of one hundred seeds (W100 in g), plant height (height) and number of nodes (Nno) assessed in AHAS genotypes treated and untreated with imazapyr derived from the BR-16 and Doko soybean cultivars sown in December 2001 in a greenhouse

Sources of variation	df	Np	Ns	Weight	Nsp	W100	Height	Nno
Genotype (G)	1	7630.0 **	21047.1 **	294.5 **	1.45 **	457.8 **	12093.3 **	39.8 **
Treatment (T)	1	391.1 *	970.0	8.33	0.43 *	365.6 **	24921.3 **	53.0 **
G x T	1	83.3	1.08	0.99	0.71 **	62.7 **	387.8 **	5.24 **
Position (P)	2	275.5 *	1615.7 **	33.0 *	0.50 **	20.5 **	_	_
G x P	2	1765.0 **	6503.4 **	202.3 **	0.09 *	12.1 *	_	_
T x P	2	1042.8 **	5414.1 **	163.1 **	0.12 *	9.30	_	_
G x T x P	2	197.4 *	989.5 *	67.5 **	0.02	0.56	_	_
Error	144	64.8	283.1	10.1	0.03	3.18	36.9[1]	0.26[1]
Mean		27.1	52.8	9.64	1.97	18.9	94.1	13.0
CV(%)		29.8	31.9	33.0	8.26	9.46	6.4	3.9

[1]df for mean square deviation was 48. **; * Significant at a probability level of 1% and 5%

Table 4. Means of the number of pods (Np), number of seeds (Ns), grain weight (W in g), number of seeds per pod (Nsp), and weight of one hundred seeds (W100 in g) obtained in the upper, mid and lower position of the plants and on the whole plant (general) in AHAS genotypes treated and untreated with imazapyr, derived from the BR-16 and Doko soybean cultivars sown in December 2002 in a greenhouse

Positions	BR-16-AHAS					Doko-AHAS				
	Np	Ns	W	Nsp	W100	Np	Ns	W	Nsp	W100
					Upper					
untreated	21.96 a[1]	46.5 a	8.96 a	2.13 a	19.48 b	36.88 a	71.65 a	11.58 a	1.95 a	16.18 b
treated	9.46 b	21.35 b	4.99 b	2.22 a	23.90 a	35.27 a	64.96 a	12.06 a	1.85 a	18.55 a
					Mid					
untreated	24.58 a	48.65 a	8.91 a	1.96 b	18.20 b	44.35 a	87.04 a	13.57 a	1.94 a	16.04 b
treated	13.77 b	31.62 b	6.95 a	2.18 a	23.15 a	36.16 b	68.88 b	12.69 a	1.90 a	18.34 a
					Lower					
untreated	20.58 b	36.19 b	6.48 b	1.75 b	17.74 b	23.46 a	41.85 a	6.96 a	1.78 a	16.71 a
treated	30.00 a	62.92 a	13.31 a	2.08 a	21.21 a	28.19 a	52.23 a	9.22 a	1.76 a	17.43 a
					General					
untreated	22.37 a	43.78 a	8.12 a	1.95 b	18.47 b	34. 90 a	66.85 a	10.70 a	1.89 a	16.31 b
treated	17.74 b	38.63 a	8.42 a	2.16 a	22.80 a	33.19 a	62.03 a	11.33 a	1.86 a	18.10 a

[1] Treatment means followed by the same letter do not differ in the F test at 5% probability

Table 5. Mean height and number of nodes (Nno) in AHAS genotypes treated and untreated with imazapyr derived from BR-16 and Doko soybean cultivars assessed in sowings in December 2001 in a greenhouse

AHAS genotypes	BR-16-AHAS		Doko-AHAS	
	Height	Nno	Height	Nno
Untreated	83.00 a[1]	12.81 a	118.96 a	15.19 a
Treated	74.62 b	11.42 b	99.65 b	12.54 b

[1] Treatment means followed by the same letter do not differ in the F test at 5% probability

experiments, so that the perception of the herbicide effects was a little different, especially for the most affected position of each genotype. For example, Doko was most affected in the mid third in the summer, while in the winter this effect was detected in the upper third. However, the responses for the two genotypes were similar in both environments. The greater ease in visualizing the herbicide effects in the winter indicates that tolerance assessments should be realized in this period, observing pod production in the upper plant parts.

CONCLUSIONS

The herbicide imazapyr applied at 100g i.a. ha^{-1} on soybean plants transformed with the *AHAS* gene can cause the formation of sterile pods, mainly on nodes of the upper plant parts. The effect occurs because of the herbicide and not because of the inserted gene and is more pronounced under winter conditions that are characterized by lower temperatures and shorter photoperiods. The magnitude of this effect is greater in BR-16 but little evident in Doko. Field experiments need to be carried out to ascertain whether there is a risk of losses in the commercial cropping situation.

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Comportamento de genótipos de soja transformados com o gene *AHAS* que confere tolerância ao herbicida imazapyr

RESUMO - *A transformação das cultivares de soja BR-16 e Doko com o gene* AHAS *resultou no desenvolvimento de genótipos tolerantes ao herbicida imazapyr. Visando comparar genótipos BR-16 e Doko convencionais e transformados, tratados com esse herbicida, instalou-se um experimento em casa-de-vegetação em condições de inverno. Após avaliação dos caracteres (número de vagens e peso de sementes) observou se que o genótipo BR-16-AHAS foi mais afetado do que Doko-AHAS, quando comparados às respectivas cultivares convencionais. Outro experimento foi instalado apenas com os genótipos AHAS com aplicação de imazapyr nas doses 0 e 100 g i.a.* ha^{-1} *durante o verão. As respostas relativas dos dois genótipos foram similares àquelas obtidas no inverno, porém a percepção do efeito do herbicida diferiu quanto à posição mais afetada em cada genótipo. O herbicida imazapyr, aplicado na dose de 100 g i.a.* ha^{-1} *sobre plantas transformadas com o gene* AHAS, *pode promover a formação de vagens estéreis. Este efeito ocorre em função do herbicida e não do gene inserido.*

Palavras-chave: *Arabidopsis thaliana*, organismos geneticamente modificados-OGM, efeito teratogênico, fitotoxicidade, interação genótipo x ambiente.

REFERENCES

Aragão FJL, Sarokin L, Vianna GR and Rech EL (2000) Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merril] plants at a high frequency. **Theoretical and Applied Genetics 101**: 1-6.

Beardmore RA, Hart R, Iverson R, Risley MA and Trimmer M (1991) Imazapyr herbicide. In: Shaner DL and O'Connor SL (eds.) **The imidazolinone herbicides**. CRS Press, Florida, p. 211-227.

Gasser CS and Fraley RT (1989) Genetically engineering plants for crop improvement. **Science 244**: 1293-1299.

Padgette SR, Kolacz KH, Delannay X, Re DB, Lavallee BJ, Tinius CN, Rhodes WK, Otero YI, Barry GF, Eichholtz DA, Peschke VM, Nida DL, Taylor NB and Kishore GM (1995) Development, identification and characterization of a glyphosate-tolerant soybean line. **Crop Science 35**: 1451-1461.

Vidal RA (1997) **Herbicidas: mecanismos de ação e resistência de plantas**. Palotti, Porto Alegre, 165p.