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Research Article
Genetic Fidelity Testing of Soybean Plants under Allelopathic Stress of *Eucalyptus* Ground Leaves Through RAPD and ISSR Markers

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Abstract

Background and Objectives: In agroforestry system (e.g., *Eucalyptus* forestry), plant-plant chemical interactions can have a strong impact on the biodiversity and dynamics of these ecosystems. The objective of the study was to evaluate the genetic stability of crop plants under allelopathic stress of tree litter. PCR based analysis system (RAPD and ISSR) was employed to determine molecular markers associated with allelopathic tolerance in *Glycine max*. **Materials and Methods:** Pot experiment was conducted with soybean seeds using mixture of soil and *Eucalyptus* ground leaves (EUGL) in a percentage of 0 (control), 10, 20, 30, 40, 50, (w/w). Leaves were harvested after 3 weeks for DNA extraction and further PCR assays. Genetic fidelity testing was assessed by calculations of genome template stability index (GTS). **Results:** Collectively, 18 new DNA markers were detected by both RAPD and ISSR analysis, in EUGL exposed plants. The RAPD and ISSR profiles verified a general tendency of decrease in GTS values with exposure to EUGL in a dose-dependent manner. A drastic decrease in GTS was more pronounced by RAPD markers, compared to ISSR. Low estimated GTS values for EUGL plants reflect their high genetic instability. **Conclusion:** The RAPD seven markers and ISSR 11markers was proved as reliable molecular markers for allelopathy tolerance in *Glycine max* and could be used in breeding programs. Moreover, lowest estimated GTS values for EUGL plants reflect their high genetic instability.

Key words: *Eucalyptus* ground leave (EUGL), *Glycine max*, ISSR, RAPD, genome template stability, allelopathy, molecular markers

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean (*Glycine max* L. Merril), an annual legume crop, is known as the first crop among the oilseed crops all over the world. It contributes by 50% of oilseed production among all oilseeds crops[1]. Furthermore, soybean is a major source of protein for human consumption and animal feed[2]. To produce a high-yielding soybean crop, genetic resources should be tested for productivity, quality parameters and stress tolerance. Because soybean varieties have different levels of adaptability to various environments, countries have different cultivars GenBanks. Therefore, each country organizes different breeding programs to create new improved cultivars. Upon increased demand on high yield varieties, there is a requirement to introduce new genotypes to select breeding materials. Therefore, it becomes necessary to ensure the quality of the plants by evaluation of their genetic consistency through cytological or molecular markers[3]. Various techniques have been used to establish the genetic fidelity and to confirm genetic homogeneity of the tested plants, such as isozymes, cytological and molecular markers[4]. However, ISSR and RAPD markers are widely tested to elucidate genetic uniformity and quality of the plants and for screening genotypes and to monitor the genetic fidelity of plants exposed to environmental stressors[5-8]. On the other hand, genetic mutations induced by environmental factors being exceptionally successful in changing seed oil composition of several oil seed crops[9]. In addition, they are considered as a source of increasing genetic variability and improvement of various agronomic traits such as yield and stover quality as well as for abiotic and biotic stress factors resistance[10,11].

Chemical interactions exhibited by plants in agroforestry system (e.g., *Eucalyptus* forestry), can have a strong impact on the dynamics of these ecosystems, as a result of their high secondary metabolite diversity. Plant invasions and their role in plant-plant competition for resources has also been the focus of several original research articles[12-15], providing an important ecological mechanism that influences the type of existing vegetation in an ecosystem and plant biodiversity.

The PCR associated DNA molecular markers based analysis system such as RAPD and ISSR have been used for detection of polymorphism among populations and genetic resources in soybean and other plant systems[16-18]. These molecular markers are different in type of inheritance, technical principle and reproducibility, percentage of polymorphism and in their costs[19]. Banding patterns can be scored for genomic template stability (GTS) evaluation in order to detect various types of DNA damage and mutations shown by the cells of bacteria, plants and animals[20,21]. The RAPD and ISSR markers have been used also for GTS evaluation to indicate DNA fidelity/stability and studying relationship between varieties of environmental stressors and genetic diversity[22-24].

The present work aimed to: (1) Determine the molecular markers associated with allelopathic tolerance in *Glycine max*, (2) Evaluate the genetic fidelity/stability of soybean plants under allelopathic stress of *Eucalyptus* tree litter. This was achieved through molecular diagnostics of exposed soybean plants to determine its DNA-based diversity as well as the level of Genome stability using ISSR and RAPD markers.

MATERIALS AND METHODS

Experimental plants and treatments: *Eucalyptus* leaves were collected from different locations of Taif Governorate, Saudi Arabia in 2015-2016. Healthy seeds of soybean were selected and imbibed with distilled water for 48 h. Pot experiment was conducted and the seeds were planted in pots containing mixture of soil and *Eucalyptus* ground leaves (EUGL) in a percentage of 0 (control), 10, 20, 30, 40, 50, (w/w). Pots maintained in a photoperiod of 10-14 h (light/dark) and controlled temperature (20°C±2). They were irrigated with water and harvested after 3 weeks for DNA extraction and further PCR assays.

Genomic DNA extraction, ISSR and RAPD assays: About 40-100 mg of young leaves from each plant was used for DNA extraction. The extraction was carried out using a small-scale DNA isolation method, using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. DNA checked on 1% agarose gel and visualized by UV transilluminator (Biometra UV star 15). The PCR assays were carried out in a 25 µL volume using 5X Firepol Master mixes, following the manufacturer's instructions. For RAPD- PCR, four primers were selected for generation of RAPD markers (Table 1). The PCR protocol was carried out according to Nkongolo *et al.*[25]. The DNA amplifications were performed in thermocycler with a preliminary step of 3 min at 94°C, 44 cycles of 20 sec at 94°C, 40 sec at 37°C and 1 min at 72°C and a final step of 7 min at 72°C. For generation of ISSR markers, a total of six primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were selected (Table 2). The PCR protocol was carried out according to Nagaoka and Ogihara[26] and Nkongolo *et al.*[25], with some modifications. The DNA amplifications were performed in thermocycler with a preliminary step of 5 min at 94°C, 42 cycles of 90 sec at 95°C, 2 min at 55°C and 60 sec at 72°C and a final step of 7 min at 72°C.

The PCR products were visualized on 1.4% agarose gels in 1X TBE buffer running at 100 V for 1.30 h. The gels were stained using ethidium bromide and visualized by UV transilluminator (Biometra UV star 15). The gel was then photographed and documented using the GelPro32 software. To verify the band profile reproducibility, RAPD and ISSR amplifications were repeated at least three times and only the repetitive PCR products were scored.

The DNA banding patterns generated by ISSR and RAPD were analyzed by computer program GelPro32 (version 4.03). The resulting amplicons from the successfully amplified primers were documented as diallelic characters: Present = 1, absent = 0. The results of ISSR and RAPD profile were analyzed by considering the bands that appeared in the control sample as the criterion of judgment. Disappearance of bands and appearance of new bands were considered to assess any DNA alteration. For each experimental group, average of genomic template stability (GTS%) was calculated according to the formula proposed by Atienzar *et al.*[21] and Liu *et al.*[27]. To measure the informativeness of the ISSR and RAPD markers in differentiation among tested plants, polymorphism information content (PIC) as a marker discrimination power, was calculated according to the formula proposed by Ghislain *et al.*[28]:

$$PIC = 1\_ [(p)\ 2+(q)\ 2]$$

where, p is the frequency of the allele band present and q is frequency of the allele band absent at a given locus. The marker index (MI) was also calculated for each ISSR and RAPD primer as:

$$MI = PIC.\eta\beta$$

where, PIC is the mean PIC value, η is the number of bands and β is the proportion of polymorphic bands, based on the method of Powell *et al.*[29].

RESULTS

RAPD and ISSR markers analysis: Genetic fidelity analysis using genomic DNA of the untreated (control) and treated plants was carried out to confirm genetic stability using RAPD and ISSR markers. The fingerprinting profiles of the soybean plants using the RAPD and ISSR markers produced distinct and reproducible amplified products (Fig. 1) and scoring data are summarized in (Table 1, 2). All ISSR and RAPD primers detected modifications in the DNA fingerprint of the leaves from treated and untreated seeds and alterations such as gain and loss of bands were observed in both molecular markers compared to control (Table 3, 4).

It was obvious from PCR profiles that differences in the number of bands were exhibited by each primer. The number of amplified bands produced per RAPD primers and their molecular size are shown in Table 1. Of these bands, 76.2% were polymorphic and 23.8% were monomorphic across the tested plants. All RAPD primers used in this study generated unique amplified bands (8 bands) which could be used as DNA markers to distinguish the exposed plants from controls (Table 1, Fig. 1). Primers OPO-01 and OPU-16 generated three unique bands which present only in EUGL exposed plants (10 and 50%), respectively and absent in control, whereas, the other five unique bands were observed

Table 1: List of RAPD primers

Primers	Sequence (5'-3') primer	MW (bp) range	AB	MB	PB	UB	P (%)	PIC	MI
OPO-01	TGCGGGTCCT	372-1282	7	3	4	1	57.15	0.38	1.51
OPO-03	CTGTTGCTAC	205-670	4	1	3	2	75	0.36	1.08
OPK-11	AATGCCCCAG	225-785	6	0	6	3	100	0.17	1.02
OPU-16	CTGCGCTGGA	189- 764	4	1	3	2	75	0.38	1.14
Total			21	5	16	8	-	-	
Mean								0.32	1.19

AB: Number of amplified bands, PB: Number of polymorphic band, MB: Monomorphic bands, UB: Unique bands, (P%): Percentage of polymorphism, PIC: Polymorphism index content , MI: Marker index

Table 2: List of ISSR primers

Primers	Sequence (5'-3') primer	MW (bp) range	AB	MB	PB	UB	P (%)	PIC	MI
UBC-809	(AG) 8 G	128-269	4	2	2	0	50.00	0.27	0.54
UBC-810	(GA) 8 T	107-271	5	2	3	0	60.00	0.37	1.11
UBC-812	(GA) 8 A	261-567	5	1	4	1	80.00	0.10	0.40
UBC-813	(CT) 8 T	284-441	3	1	2	2	66.60	0.375	0.675
UBC-826	(AC) 8 G	261-922	8	1	7	2	87.50	0.36	2.50
UBC-828	(TG) 8C	118-759	3	1	2	0	66.60	0.45	0.891
Total			28	8	20	5		-	
Mean								0.32	0.87

AB: Number of amplified bands, PB: Number of polymorphic band, MB: Monomorphic bands, UB: Unique bands, (P%): Percentage of polymorphism, PIC: Polymorphism index content, MI: Marker index

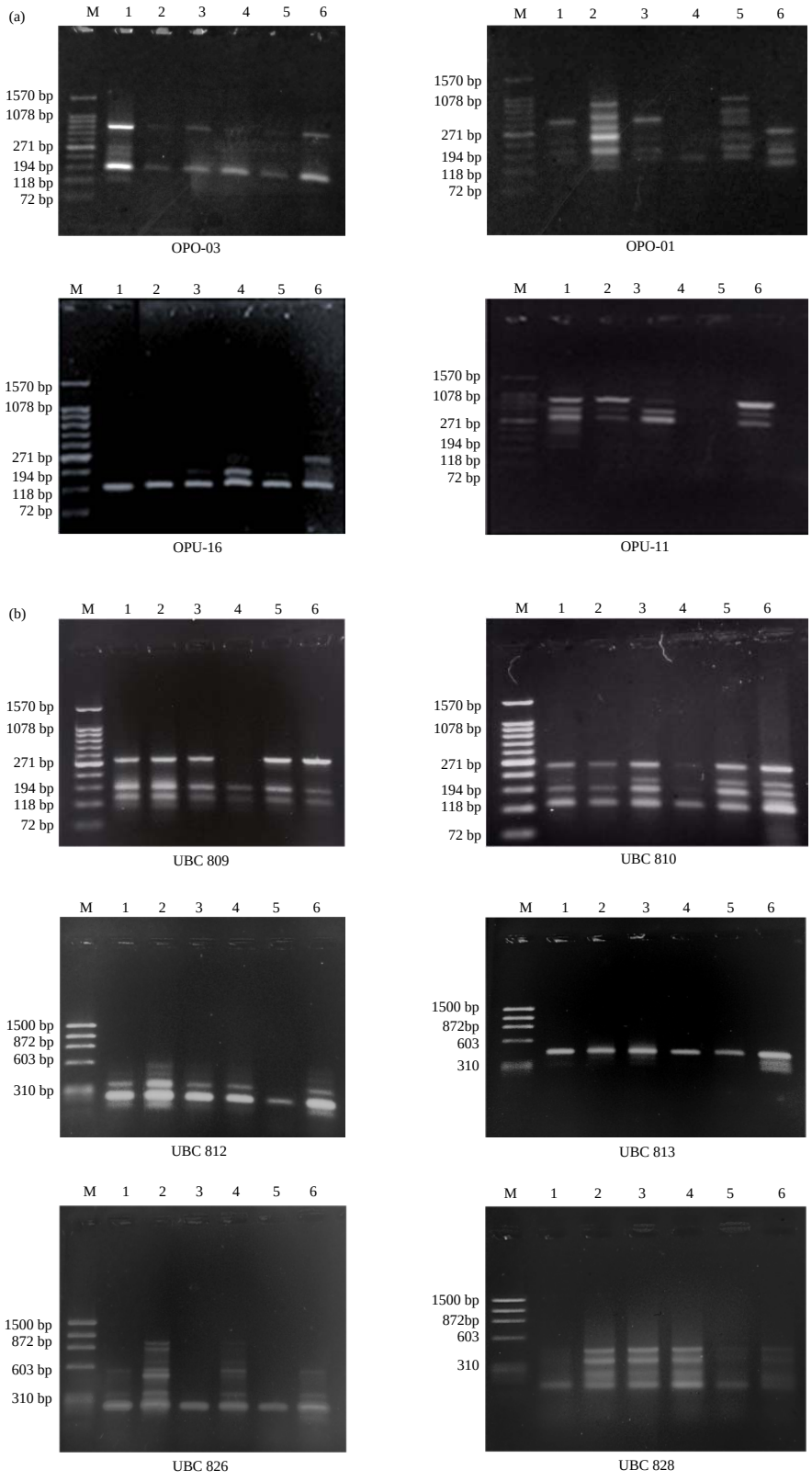

Fig. 1(a-b): (a) RAPD markers and (b) ISSR markers generated by EUGL treated soybean plants with tested primers
M: DNA marker, 1: 0% (control), 2: 10%, 3: 20%, 4: 30%, 5: 40%, 6: 50%

Table 3: Number of bands in control with all RAPD primers, polymorphic bands and polymorphism percent (P%) in EUGL exposed soybean plants

		10%		20%		30%		40%		50%	
	Control										
Primers	Total bands	a	b	a	b	a	b	a	b	a	b
OPO-01	3	3	0	2	0	0	0	3	0	1	0
OPO-03	4	0	2	0	2	0	2	0	2	0	2
OPK-11	6	0	3	0	3	0	6	0	3	0	6
OPU-16	1	0	0	1	0	1	0	1	0	2	0
Total bands	14	3	5	3	5	1	8	4	5	3	8
a+b		8		8		9		9		11	
P%		57.14		57.14		64.3		64.3		78.57	

a: Indicates appearance of new bands, b: Disappearance of normal bands, a+b: Polymorphic bands, (P%): Polymorphic percentage

Table 4: Number of bands in control with all ISSR primers, polymorphic bands and Polymorphism percent (P%) in EUGL exposed soybean plants

		10%		20%		30%		40%		50%	
	Control										
Primers	Total bands	a	b	a	b	a	b	a	b	a	b
UBC-809	4	0	0	0	1	0	2	0	1	0	1
UBC-810	3	0	0	0	0	0	1	1	0	2	0
UBC-812	4	1	0	1	0	1	0	0	3	1	0
UBC-813	1	0	0	0	0	0	0	0	0	2	0
UBC-826	3	4	0	0	2	3	0	0	2	1	0
UBC-828	2	0	0	1	1	0	1	1	1	1	1
Total bands	17	5	0	2	4	4	4	2	7	7	2
a+b		5		6		8		9		9	
P%		29.4		35.3		47.05		52.94		52.94	

a: Indicates appearance of new bands, b: Disappearance of normal bands, a+b: Polymorphic bands, (P%): Polymorphic percentage

Table 5: Molecular RAPD-PCR markers of 4 tested primers associated with EUGL exposed soybean plants and their MW

			EUGL concentrations (%)						
Primer	BN	MW	0	10	20	30	40	50	New RAPD marker
OPO-01	1	1282	0	1	0	0	0	0	+
	2	1047	0	0	1	0	1	0	+
	3	872	0	1	1	0	1	1	+
	4	613	1	1	1	1	1	1	
	5	546	0	1	0	0	1	0	+
	6	489	1	1	1	1	1	1	
	7	372	1	1	1	1	1	1	
OPO-03	1	670	1	1	1	0	0	1	
	2	275	1	0	0	0	0	0	
	3	248	1	0	0	0	0	0	
	4	205	1	1	1	1	1	1	
OPK-11	1	785	1	1	1	0	1	0	
	2	300	1	1	1	0	1	0	
	3	272	1	1	1	0	1	0	
	4	248	1	0	0	0	0	0	
	5	255	1	0	0	0	0	0	
	6	123	1	0	0	0	0	0	
OPU-16	1	764	0	0	0	0	0	1	+
	2	281	0	0	0	0	0	1	+
	3	253	0	0	0	1	1	1	+
	4	189	1	1	1	1	1	1	
Total									7

in controls and absent in EU exposed plants, two of them were revealed by OPO-03, whereas, primer OPK-11 produced three bands (Table 5). Polymorphism ranged from 57.1% at primer OPO-01 to100% at primer OPK-11. The least values of PIC index and MI were exhibited by OPK-11 whereas, primer OPO-1 revealed the highest values (Table 1). All RAPD primers exhibited the generation of 7 new markers (Table 5).

Table 6: Molecular ISSR-PCR markers of 4 tested primers associated with EUGL exposed soybean plants and their MW

			EUGL concentrations (%)						
Primer	BN	MW	0	10	20	30	40	50	New ISSR markers
UBC-809	1	269	1	1	1	0	1	1	
	2	202	1	1	1	1	1	1	
	3	131	1	1	1	1	1	1	
	4	128	1	1	0	0	0	0	
UBC-810	1	271	1	1	1	1	1	1	+
	2	245	0	0	0	0	1	1	
	3	184	1	1	1	0	1	1	
	4	162	1	1	1	1	1	1	
	5	107	0	0	0	0	0	1	+
UBC-812	1	567	0	1	1	1	0	1	+
	2	462	1	1	1	1	0	1	
	3	340	1	1	1	1	0	1	
	4	274	1	1	1	1	1	1	
	5	261	1	1	1	1	0	1	
UBC-813	1	441	1	1	1	1	1	1	
	2	366	0	0	0	0	0	1	+
	3	284	0	0	0	0	0	1	+
UBC-826	1	922	0	1	0	1	0	0	+
	2	831	0	1	0	0	0	0	+
	3	684	0	1	0	0	1	0	+
	4	556	1	1	0	1	0	1	
	5	498	0	1	0	1	0	0	
	6	343	1	1	0	1	0	1	
	7	296	1	1	1	1	1	1	
	8	261	0	0	0	0	0	1	+
UBC-828	1	759	0	0	1	0	1	1	+
	2	246	1	1	1	1	1	1	
	3	118	1	1	0	0	0	0	
Total									11

The ISSR primer sets utilized to analyze soybean seedlings generated approximately an average of 4.7 bands per primer. 71.4% of amplified bands were polymorphic and 28.6% were monomorphic (Table 2). The highest percentage of polymorphism (%P) was generated by UBC-826, while the lowest was generated by UBC-809. Out of 6 ISSR primers used in this study, 3 of them generated 5 unique amplified bands, which present only in EUGL exposed plants and absent in control (Table 4, Fig. 1). Primer UBC-810 generated 1 unique band and two bands were also revealed by UBC-813 at high concentration whereas primer UBC-826 produced 2 unique bands at both lower and higher concentrations. Collectively, 11 new ISSR markers were developed in treated plants (Table 6). UBC-828 showed the highest PIC, whereas the lowest value revealed by UBC- 813. On the other hand, lower MI value for ISSR, was revealed by UBC-813 though UBC-826 showed the higher one (Table 2).

The EUGL presented more alterations by RAPD analysis and higher concentrations (30, 40 and 50%) revealed the highest total number of alterations with this marker, of the 45 altered bands from all evaluated individuals, 68.88% corresponded to loss and 31.11% to gain of a band. (Table 3). Differently, in ISSR marker analysis, the most commonly occurring type of alteration was the gain of bands, EUGL drove a 54.05% gain and 45.9% loss of bands (Table 4).

Aimed at verifying genetic effects of allelopathy, presented by EUGL exposure, alterations in the ISSR and RAPD patterns were expressed as decreases in GTS, a qualitative measure reflecting the change in the number of amplicons generated by EUGL treatment, in relation to profiles obtained from the non-treated plants. The GTS (%) was calculated for each primer and presented in Fig. 2. The results exhibited a general tendency of decrease in GTS values with exposure to EUGL. Genome stability tended to be reduced strongly in case of EUGL higher concentrations. A drastic decrease in genome stability was more pronounced by RAPD markers, compared to ISSR.

DISCUSSION

The DNA-based molecular markers are essential genomic resources to accomplish genetic studies or marker-aided breeding in any crop. In *Glycine max*, various DNA-based molecular markers such as AFLP, RFLP, RAPD, ISSR expressed

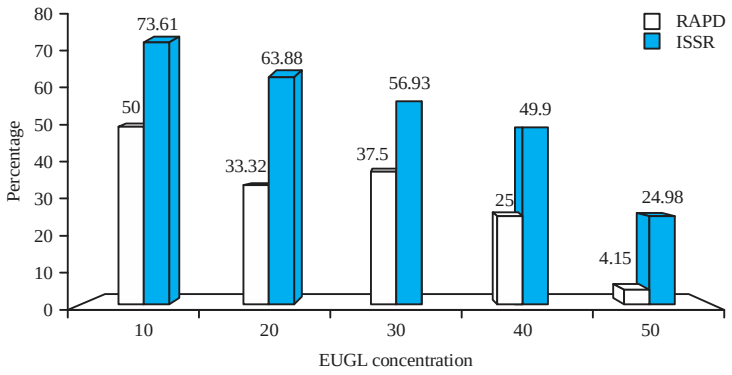

Fig. 2: Comparison of genomic DNA stability (GTS) in EUGL treated soybean plants with tested RAPD and ISSR markers

sequence tag-based (EST) markers, sequence-tagged sites (STSs), simple sequence repeat (SSRs/microsatellites), have been developed to distinguish genetic variability, linkage map analysis and marker assisted screening to expedite the breeding programs[30-32,4].Consequently, *Glycine max* has substantial stock of genetic and genomic resources in the form of DNA-based markers, mapping populations or linkage maps. This study was designed to explore the genetic response of soybean genome to the allelopathic effects of *Eucalyptus* ground leaves (EUGL) using molecular-diagnostics approach via molecular markers. Therefore, understanding how soybean responds to plant canopy situations is imperative for breeding towards tolerance to allelopathy. Toward these aims, powerful genetic and molecular technologies have been used to identify soybean genes playing essential roles in plant resistance to allelopathy environments[15]. It has been suggested that use of multiple markers amplifying various regions of the genome, allows high likelihood for the successful identification of polymorphism[33,34]. The RAPD and ISSR usually based on the non-coding regions of DNA, are simple, cost effective and proved efficient in evaluating the genetic homogeneity, genetic diversity and evolutionary studies[35,36]. Both markers have been successfully applied to determine genetic fidelity/stability in several plant systems e.g., sugarcane[16], *Chlorophytum borivilianum*[37], *Terminalia bellirica*[38], *Aloe barbadensis*[39]. Data analysis of RAPD and ISSR profiles identified 18 new bands in soybean plants grown under EUGL effect. These bands might be considered as useful markers linked with allelopathy tolerance in soybean.

In this study, RAPD and ISSR DNA markers were also employed to examine the instability of the soybean genome after exposure to EUGL. Both RAPD–PCR and ISSR-PCR profile in EUGL-treated plants verified extensive differences between untreated and treated plants, in a dose-dependent manner. Assessment of genetic stability was based on a qualitative measurement of the changes in ISSR and RAPD profiles generated by EUGL-exposed soybean plants, which is expressed as a decrease in GTS, in relation to the profiles obtained from the control plants. The results analysis showed a dose-dependent reduction in GTS%, verifying genetic instability of the *Glycine max* genome as exposed to EUGL allelochemicals. This finding is congruent with a previous study which documented the genome instability in allelopathy-exposed crops[40], suggesting the risk of the by-products of allelopathic plant species. Overall, low estimated GTS values combined with the high polymorphism level reported for EUGL treated plants could explain their allelopathy tolerance compared to the untreated plants. The decrease in GTS could be attributed to genetic variation, inducing new protein in relation to allelopathy tolerance[41].

The main changes in the RAPD and ISSR profiles of the present investigation were pronounced as changes in the number and position of the bands between the treated and untreated plants. The loss and gain of bands might be due to the structural re-arrangements in DNA caused by different types of DNA damages, such as DNA adducts, breakages and mutations at the annealing site with consequent alterations of the DNA fingerprints[42]. These effects seem to be correlated with the level of damage in the DNA template after exposure to allelochemicals exerted by EUGL. Thus, the recorded gain or loss alterations in this study proof the genome in stability of the soybean genome in regard to *Eucalyptus* allelo chemicals. This instability is brought about by mutations, particularly those that damage the cell maintenance in processes of DNA repair, replication and recombination, as well as cell division[43]. The highest loss of bands was verified with the RAPD marker. This probably due to alterations in heterozygote loci of the

genome, generated by allelochemicals. The loss of bands can be more easily verified in heterozygous loci. In contrast, the highest gain of bands was observed in the ISSR analysis. The appearance of bands could be attributed to the formation of new sites for primer annealing by point mutations, or chromosome rearrangements forming sequences complementary to the primer sequence, generating new alleles[44]. High polymorphism of ISSR and RAPD markers was also reported in many previous studies[45,46]. In the present work, high levels of polymorphism exhibited by the PIC value of both markers indicated the usefulness of them for germplasm evaluation[47]. Moreover, they both presented equal effectiveness in allelopathic potential evaluation which could be further employed for identification of the target genes of the allelopathic agents.

CONCLUSION

Allelopathy stress is one of the major obstacles to plant crops productivity. Various genomics tools have facilitated characterization of soybean germplasm so that potential sources of genetic variation could be incorporated. To develop crop plants with enhanced tolerance of allelopathy stress, the present study used RAPD and ISSR to identify markers associated with allelopathy tolerance. The RAPD-PCR generated seven markers, while ISSR exhibited 11 markers. Those markers could be considered as reliable molecular markers for allelopathy tolerance in *Glycine max* and could be used in breeding programs. Moreover, lowest estimated GTS values for EUGL plants reflect their high genetic instability.

SIGNIFICANCE STATEMENTS

This study highlights an important ecological problem within the agroforestry system focusing on the molecular impacts on crop plants. It is one of the few studies which employed molecular markers for evaluation of allelopathic impacts on the crop plants. This was achieved through molecular diagnostics of exposed soybean plants to determine its DNA-based diversity as well as the level of genome instability using ISSR and RAPD markers. High levels of polymorphism exhibited by both markers indicated the usefulness of them for germ plasm evaluation. Moreover, they both presented equal effectiveness in allelopathic potential evaluation which could be further employed for identification of the target genes of the allelopathic agents. This study may help to develop crop plants with enhanced tolerance of allelopathy stress.

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