

Cytogenetic and molecular studies on two faba bean cultivars revealed their difference in their aluminum tolerance

Ahmed M. HASSANEIN^{1,2}, Ahmed H. MOHAMED³, Heba Ahmed ABD ALLAH³, Hoida ZAKI³

Received November 19, 2019; accepted October 26, 2020.
Delo je prispelo 19. novembra 2019, sprejeto 26. oktobra 2020.

Cytogenetic and molecular studies on two faba bean cultivars revealed their difference in their aluminum tolerance

Abstract: Two cultivars of faba bean (*Vicia faba* 'Giza 843' and 'Nobaria 3') that differ in aluminum (Al) tolerance were used to study cytogenetic and genomic alterations under the influence of Al Cl₃ (5, 15, and 25 mmol AlCl₃) for different periods (6, 12 and 24 h). Under Al treatments, mitotic index in both cultivars decreased and total chromosomal abnormalities increased. The frequencies of micronuclei and chromosomal abnormalities (C-anaphase, metaphase-star chromosomes, breaks, sticky and disturbed chromosomes during metaphase or anaphase) in 'Giza 843' were lower than in 'Nabaria 3'. Increase of the registered cytogenetic events under the influence of Al stress led to increase the detected polymorphism using RAPD and ISSR markers. Application of RAPD primers gave the same value of polymorphism in both faba bean cultivars under Al stress. Polymorphism average of nine ISSR primers of 'Giza 843' (65.36 %) was lower than that of 'Nobaria 3' (71.59 %). Molecular markers, cytogenetic characteristics and seedling growth data indicate that Al tolerance of 'Giza 843' was higher than of 'Nobaria 3'. This work shows that cytogenetic and ISSR techniques could be used efficiently to distinguish between the ability of two faba bean cultivars to tolerate toxic effects of Al.

Key words: aluminum tolerance; *Vicia faba* L.; chromosomal abnormalities; cytogenetics; RAPD, ISSR

Citogenetske in molekularne raziskave dveh sort boba so odkrile njuno različno toleranco na aluminij

Izvleček: Dve sorti boba (*Vicia faba* 'Giza 843' in 'Nobaria 3'), ki sta se razlikovali v toleranci na aluminij (Al) sta bili uporabljeni v raziskavi citogenetskih in genomskih sprememb, ki so jih povzročile različne koncentracije Al Cl₃ (5, 15, in 25 mmol AlCl₃) v različnih časovnih obdobjih (6, 12 in 24 h). Pri obravnavanjih z Al se je mitotski indeks obeh sort zmanjšal, celokupne kromosomske aberacije pa so se povečale. Pogostost malih jeder (micronuclei) in kromosomskih aberacij (C-anafaze, zvezdasti kromosomi v metafazi, lomi kromosomov, zlepljeni in nenormalni kromosomi v metafazi in anafazi) sta bili pri 'Giza 843' manjši kot pri 'Nabaria 3'. Povečanje ugotovljenih citogenetskih dogodkov zaradi vpliva Al stresa je povečalo ugotovljeni polimorfizem z RAPD in ISSR označevalci. Uporaba RAPD začetnih oligonukleotidov je dala enako vrednost polimorfizma pri obeh sortah izpostavljenih aluminijevemu stresu. Poprečje polimorfizma devetih ISSR začetnih oligonukleotidov je bilo pri 'Giza 843' (65,36 %) manjše kot pri 'Nobaria 3' (71,59 %). Molekulski označevalci, citogenetske značilnosti in parametri rasti sejank so pokazali, da je tolerance na aluminij pri 'Giza 843' večja kot pri 'Nobaria 3'. Raziskava kaže, da bi se citogenetske metode in ISSR tehnike lahko učinkovito uporabljale za razlikovanje sposobnosti tolerance različnih sort na toksične učinke Al.

Ključne besede: toleranca na aluminij; *Vicia faba* L.; kromosomske aberacije; citogenetika; RAPD; ISSR

¹ Sohag University, Faculty of Science, Central Laboratory of Genetic Engineering, Sohag, Egypt

² Corresponding author, e-mail: Hassaneinam2@yahoo.com

³ South Valley University, Faculty of Science, Botany and Microbiology Department, Qena, Egypt

1 INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important legume plants where it is cultivated for humans as well as animals. Faba bean seeds contain high proteins and other important components. It is a diploid species with $2n = 12$. It belongs to *Viceae* tribe of Papilionoideae subfamily of Fabaceae family. In general, legume plants are used to improve the fertility of soil through nitrogen fixation. Around the world, 37 collections including about 38,360 faba bean germplasm have been conserved; the largest collection belongs to International Center for Agricultural Research in the Dry Areas - ICARDA in Syria (Duc et al., 2010).

Genetic variation in faba bean ranges from 10 to 60 % depending on plant genotype and the environmental conditions (Suso et al., 2006). Faba bean is cultivated under several conditions, but water deficiency, increased soil salinity and pollution with toxic metals such as aluminum (Al) limit the faba bean production (Abdelhamid et al., 2010). Consequently, faba bean growth and yield retardants should be investigated.

Aluminum abundance in the earth's crust is very high. In nature, the highest amount of Al is present in insoluble form (aluminum silicate), but very small amount of Al is present in soluble form; this enters and affects biological systems (May and Nordstrom, 1991). The detected negative effects of Al on plant root tips were attributed to increase of cell wall rigidity, decrease of cell respiration and DNA replication, and interfere with cell division (Llugany et al., 1995). Some authors confirmed that root tips play a major role in Al toxicity/protection (Bennet and Breen, 1991), but others refuse this hypothesis (Ryan et al., 1993). In Al-sensitive maize cultivar, Al was accumulated in the distal transition zone of the root apex (1-2 mm) and inhibited root elongation (Sivaguru and Horst, 1998).

In plants, Al tolerance varies between species or cultivars of each one and it depends on detoxification of accumulated Al or prevention of Al uptake (Rengel, 1996). Aluminum toxicity is usually associated with chromosomal changes, which can be detected by karyologic analysis of Al-treated root tips by estimating mitotic indices (MI), micronucleus (MN) formation and chromosomal abnormalities (CA) (Yi et al., 2010; Kotelnikova et al., 2019). Faba bean could be used as a test species for monitoring the genotoxicity of Al because of its small number of chromosomes and their length make it easy to detect the damage occurring under the influence of the studied factor (Gopalan, 1999; Yi et al., 2010; Altwaty et al., 2016).

Induction of reactive oxygen species (ROS) formation under Al toxicity was reported (Rout et al., 2001)

causing single and double strand DNA breaks. Consequently, the frequency of CA and MN is increased (Yi et al., 2010). In faba bean root tips, high concentrations of Al resulted in decreased MI, reduced mitotic activity and blocked DNA synthesis (Mohanty et al., 2004), and DNA recombination (Achary and Panda, 2010). Yi et al. (2010) reported that some mitotic cells could enter and continue mitotic cell division with abnormal DNA.

Chromosomal abnormalities due to abiotic stress agent could be detected not only by cytogenetic approach but also with molecular testing based on PCR. Chromosomal abnormalities as well as point mutations may result in alterations either at or between the RAPD or ISSR primer binding sites leading to alterations of the PCR products. These products can be used to calculate polymorphism which gives clear indication about the stability of the studied genome under the influence of the studied agent (Hassanein et al., 2018).

The data available so far on the toxic effects of high concentrations of Al on plant species are few. While relatively high concentrations of $AlCl_3$ was used in our study (5- 25 mmol for 24 h), they were lower than what used by others (Karimai and Poozesh, 2016). Under relatively high concentrations of $AlCl_3$, studies linking results of cytogenetical and molecular techniques are scarce. Consequently, the aim of this study was to investigate genome stability of two faba bean cultivars under the influence of relatively high dose of $AlCl_3$ using cytogenetical and molecular techniques. Also, to know which of the easiest and cheapest molecular biology techniques, such as RAPD or ISSR, confirm the obtained cytogenetic data.

2 MATERIAL AND METHODS

In preliminary experiment, seeds of ten faba bean cultivars were obtained from Agriculture Research and Seeds Center in Qena, Egypt. Faba bean seeds were surface sterilized with 5 % (v/v) commercial sodium hypochlorite for 5 min followed by 0.2 % (w/v) mercuric chloride for 5 min. Seeds were rinsed in sterilized deionized water 3 times, 10 min each. For seed germination, they were subjected to a series of $AlCl_3$ concentrations (5, 10, 15, 20, 25, 30, 35 and 40 mmol) for different exposure times (6, 12 and 24 hr). After ten days, seed germination and seedling growth parameters were estimated. Consequently, three $AlCl_3$ concentrations (5, 15 and 25 mmol) as well as the most tolerant ('Giza 843') and sensitive ('Nobaria 3') cultivars were used for further studies.

Seeds of 'Giza 843' and 'Nobaria 3' were selected to be homogeneous in size and color, and without wrinkles. Ten sterilized seeds were grown in sterilized petri dish contains filter paper and a thin layer of sterilized distilled

water (15 ml) for 2 to 3 days. Seedlings with root length of 2-3 cm were immersed in solution of $AlCl_3$ of three concentrations (5, 15 and 25 mmol) for three time periods (6, 12 and 24 h). Faba bean seeds treated with distilled water were used as control. In each treatment, three replicates were used, each with 10 seeds/Petri dish. Seeds were germinated under lab condition at 28 ± 2 °C. For each treatment, ten root tips were cut and fixed overnight in ethanol:acetic acid 3:1 (v/v) at 4 °C. Then, root tips were hydrolyzed with 1 N HCl, stained microscopic slides were prepared by at 60 °C for 8-10 minutes. Then, roots were washed and transferred into the Feulgen stain for one hour. Root tips were teased out on clean slide, coated with egg-albumen, in a drop of 45 % glacial acetic acid and they squashed between cover and slide. To separate covers, slides were turned face down in a Petri-dish containing distilled water. Plant materials were dehydrated using a series of ethyl alcohol (50 %, 70 %, 96 %) for 10 minutes in each alcohol concentration. The slides were immersed in a solution of absolute ethanol and xylol (1:1). Slides were cleared in pure xylol for 10 minutes, mounted in Canada balsam and transferred to dry in a hot air oven at 35-40 °C for 2-3 days (Darlington and La Cour, 1976; Kanaya et al., 1994).

The percentage of MI, phase indexes, total abnormalities and the percentage of the aberrations were determined according to the following formulas:

$$\text{Mitotic index (M.I.)} = (\text{No. of dividing cells} \times 100) / (\text{No. of non dividing cells} + \text{No. of dividing cells})$$

$$\text{Total abnormalities} = (\text{No. of abnormal dividing cells} \times 100) / (\text{No. of dividing cells})$$

$$\% \text{ of the phase} = (\text{No. of dividing cells in phase} \times 100) / (\text{No. of dividing cells})$$

$$\text{The percentage of the aberrations} = (\text{No. of aberrant cells} \times 100) / (\text{No. of dividing cells})$$

To determine the genetic variation and genome stability of root tips treated with 5 mM $AlCl_3$ for different periods, the RAPD and ISSR techniques were used. DNA extraction was carried out three times of each treatment and according to the method of Porebski et al. (1997). Hexadecyl trimethyl ammonium bromide (CTAB) procedure based on the protocol of Porebski et al. (1997) was used. Frozen root tissues (200 mg) were ground using 1000 μ l of CTAB buffer. The obtained homogenate was mixed gently with 2 μ l of RNase, incubated at 65 °C for 90 min and centrifuged at 10000 rpm for 5 min. Supernatant was transferred to a new tube, where 500 μ l of 24:1 chloroform-isoamyl alcohol were added and mixed well by shaking. The mixture was centrifuged at 10000 rpm for 5 min, and the aqueous phase (top) was quickly measured and isolated. Then, one volume of cold isopropanol was added. Samples were let to sit at -20 °C for 45-60 min. Samples were centrifuged for 10 min at 12000 and supernatant was carefully removed without disturbing the resultant DNA pellet. Then, 700 μ l of cold 70 % ethanol were added, inverted once to mix and centrifuged for 1 min at 12000 rpm. The DNA pellet was dried at 55 °C, resuspended in 100 μ l of TE buffer and allowed to resuspend for 1 hr at 55 °C before use.

A total of ten RAPD and nine ISSR primers (Table 1) were used to analyze genome stability of Al treated roots. The obtained genomic DNA was amplified using Thermal Cycler (Biometra TPersonal Combi, Biometra GmbH, Germany). The RAPD and ISSR reactions were performed in a 25 μ l volume containing 12.5 μ l of Go Taq® Green Master Mix (Promega, Madison, USA), 3 μ l of primer 10 pmol, 6.5 μ l of free nuclease water and 3 μ l of 100 ng genomic DNA templates.

Table 1: The applied RAPD and ISSR primers

RAPD Primer	Primer Sequence (5'-----3')	ISSR Primer	Primer Sequence (5'-----3')
OPA-02	TGCCGAGCTG	ISSR1	ACACACACACACACACCTG
OPA-05	AGGGGTCTTG	ISSR2	CACACACACACACAAAGCT
OPA-07	GAAACGGGTG	ISSR3	ACACACACACACACAAG
OPA-17	GACCGCTTGT	ISSR4	GAGAGAGAGAGAGACTG
OPat-08	TCCTCGTGGG	ISSR5	GAGAGAGAGAGAGACTC
OPaw-10	GGTGTTTGCC	ISSR7	CTCTCTCTCTCTA (CT)6A
OPD-1	ACCGCAAGG	ISSR8	TCTTCTTCTTCTG
OPD-18	GAGAGCCAAC	ISSR9	TGTTGTTGTGC
OPJ-15	TGTAGCAGGG	ISSR10	GTGGTGGTGGC
OPP-13	GGAGTGCCTC		

The program of PCR amplification was applied with initial denaturation cycle at 94 °C for five minutes. The next 40 cycles were carried out using denaturation step at 94 °C for 45 sec, annealing step (optimized for each primer), and elongation step at 72 °C for 1 min. Finally, extension step was performed at 72 °C for 7 min. The obtained PCR products were subjected to electrophoresis using 1.5 % or 2 % agarose gel with ethidium bromide (0.5 µg ml⁻¹). Run was carried out in 1X TBE buffer at 70 volts. The amplified PCR products were visualized and photographed. Polymorphic, monomorphic, unique, total and molecular weight of each band were determined.

Reproducibility was taken in consideration to minimize personal errors. In this concern, each primer was used three times under the same PCR conditions. The DNA banding patterns obtained from amplification of the studied genome using RAPD and ISSR primers were analyzed by a computer program; Gene Profiler (version 4.03). Microsoft excel file was prepared for scoring the data as '1' for matched and '0' for the unmatched of DNA band. Monomorphic, polymorphic and unique bands were taken into consideration to calculate polymorphism of each primer to determine the number of events leading to alteration of primer binding sites on the genome under the toxic effect of Al compared to control plants. Dendrograms were generated for cluster analysis according to Legendre and Legendre (1983) using the Community Analysis Package Software Program (CAP) Version 4.0 (Richard and Peter, 2007).

3 RESULTS

Depending on data of preliminary seed germination and seedling growth, 'Giza 843' was found to be the most tolerant but 'Nobaria 3' was the most sensitive faba bean cultivar to AlCl₃. Cultivar 'Nobaria 3' recorded the highest decrease of root growth parameters, where they were 5.71 %, 74.29 % and 77.14 % for root lengths, 53.41 %, 80.68 % and 87.50 % for fresh mass, and 50 %, 75 % and 75 % for dry mass under the treatment with 5, 15 and 25 mmol AlCl₃, respectively. Under these conditions, 'Giza 843' recorded increased fresh (6 %) and dry mass (22 %) but reduced shoot length (3.3 %) when 5 mmol AlCl₃ was used. In addition, 'Giza 843' showed reduced root growth parameters where they were 68.85 % and 72.13 % for root length, 53.76 % and 68.82 % for fresh mass, and 44.44 % and 66.67 % for dry mass of 15 and 25 mmol AlCl₃, respectively.

Aluminum chloride caused a significant decrease in the mitotic activities in treated root tips of 'Giza 843' or 'Nobaria 3' cultivars (Tables 2 and 4) when they were treated with different concentrations of AlCl₃ for dif-

ferent time periods. Data of MI indicate that 'Giza 843' was the least affected when root tips were subjected to different concentrations of AlCl₃ for 6 hr. For example, under the influence of the lowest concentration of AlCl₃ (5 mmol) and the shortest exposure time (6 h), MI of 'Giza 843' (2.70) was higher than that of 'Nobaria 3' (1.87). The same was observed when the highest concentration of AlCl₃ (25 mmol) was used for 6 hr. MI of 'Giza 843' (3.10) was higher than that of 'Nobaria 3' (2.60). The lowest MI values were detected when root tips of both cultivars were subjected to different Al concentrations for 24 hr. In 'Giza 843', complete inhibition of mitotic cell activities was detected when the highest concentration of Al (25 mmol AlCl₃) for the longest time of exposure (24 hr) was used.

In comparison to that of control (AlCl₃-untreated plants), the increase in AlCl₃ concentration or exposure time led to significant increase in the values of interphase and it associated with significant increase in total chromosomal abnormalities in both cultivars (Table 2 and 4). In addition, while the highest values of interphase were detected under 25 mmol Al for 24 hr, prophase stage was inhibited completely in both cultivars. In 'Giza 843', when the AlCl₃ exposure time was 6 hr, total abnormalities increased with the increase of Al concentrations. In additions, when the concentration of AlCl₃ was 5 mmol, total abnormalities increased with the increase of exposure time. Under the influence of all AlCl₃ concentrations for 6 or 12 h, total abnormalities of 'Giza 843' were lower than those of 'Nobaria 3'. While total mitosis and MI drastically decreased under AlCl₃ treatments for 24 h, high total chromosomal abnormalities were detected in both cultivars. The frequency of abnormalities in all mitotic phases increased under AlCl₃ treatments in both cultivars.

In both cultivars, the frequency of MN increased under the influence of all AlCl₃ treatments, this increase was lower in 'Giza 843' than 'Nobaria 3' (Tables 3 and 5). Cells with one, two or more micronuclei appeared in both cultivars (Fig. 1).

All AlCl₃ concentrations induced chromosomal abnormalities at all stages of mitosis; depended on the used cultivar. While the frequency of C-metaphase in 'Giza 843' was higher than 'Nobaria 3', vice versa was detected in case of C-anaphase (Table 3 and Table 5). On the other side, the frequency star chromosomes in metaphase of 'Giza 843' (Table 3) was lower than that of 'Nobaria 3', vice versa was detected during ana-telophase. In addition, under the influence of different AlCl₃ treatments, the frequency of different abnormalities was higher in 'Nobaria 3' than 'Giza 843'. For example, the frequencies of chromosomal breaks and sticky and disturbed chromosomes during metaphase or ana-telophase in 'No-

Table 2: Cytogenetic analysis of *Vicia faba* 'Giza 843' root tip treated with three concentrations of AlCl₃ for three exposure times. Abbreviations: Mitotic index (MI), total abnormalities, interphase and mitotic phases (prophase, metaphase and ana-telophase), include normal (Total) and abnormal (Abn.) mitotic phases.

Treatment	Exposure time (h)	AlCl ₃ conc.	Total mitosis	MI (%)	% Total abnormal	% Interphase			% Metaphase			% Ana-telophase		
						Total	Abn.	Abn.	Total	Abn.	Abn.	Total	Abn.	Abn.
6	Control		355	7.10±0.36	4.09±0.42	92.9	0.4	0.4	35.9	0.4	23.6	1.4	40.5	0
	5 mmol		138	2.70±0.40*	59.10±14.40*	97.2	0.6	11.8	44.6	11.8	29.7	25.7	25.7	15.8
	15 mmol		115	2.30±0.10*	74.50±9.50*	97.6	0.6	16.7	63.1	16.7	17.1	17.1	19.8	12.6
12	25 mmol		157	3.10±0.30*	76.47±0.55*	96.4	0.7	17.6	69.1	17.6	11.5	9.4	19.4	9.4
	5 mmol		53	1.07±0.25*	76.90±14.00*	99.0	0.5	28.5	32.6	28.5	28.6	24.5	38.8	22.4
	15 mmol		63	1.27±0.15*	67.73±1.05*	98.7	0.6	18.2	40.2	18.2	35.1	32.5	24.7	20.8
24	25 mmol		182	3.60±0.10*	79.47±3.55*	98.4	0.07	32.4	43.4	32.4	27.5	28.0	29.1	17.6
	5 mmol		73	1.47±0.15*	93.37±1.05*	98.5	1.4	35.5	38.7	35.5	54.8	54.8	6.5	3.2
	15 mmol		75	0.10±0.00*	100.00±0.00*	99.9	3.9	0	0	100	100	100	0	0
	25 mmol		0	0	0	0.9	0	0	0	0	0	0	0	0

* The mean difference is significant at the 0.05 level.

Table 3: Types and percentage (%) of chromosomal abnormalities in *Vicia faba* 'Giza 843' root tip treated with three concentrations of AlCl₃ for three exposure times. Abbreviations: C-metaphase (C-m), sticky (Stick), star, break, disturbed (Dist), and diagonal (Diag), bridge, free, C-anaphase (C-ana), micronuclei (MIN) and bi-nucleated cell (BN).

Treatment	Exposure time (h)	AlCl ₃ conc.	Interphase abnormalities					Metaphase abnormalities					Ana-telophase abnormalities				
			MIN	BN	C-m	Stick	Star	Break	Dist	Stick	Bridge	Dist	Diag	Free	Break	Star	C-ana
6	Control		0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
	5 mmol		0.16	0.5	19.8	1.9	2.9	0.9	1.9	1.9	0.9	0.9	1.9	5.9	0	0.9	4.9
	15 mmol		0.36	0.04	15.3	0	0	0.9	0	2.7	0	0.9	0.9	0.9	0.9	1.8	5.4
12	25 mmol		1.46	0.4	7.2	0	0	0.7	1.4	2.2	0	0	5.7	0	1.4	0	0
	5 mmol		0.17	0.2	20.4	4.1	0	0	8.2	2.1	0	4.1	0	0	0	6.1	2.1
	15 mmol		0.24	0.1	28.6	0	0	2.6	0	2.6	1.3	0	2.6	0	6.5	1.3	2.6
24	25 mmol		1.22	0	28.0	0	0	0	0	3.8	0	5.5	0	1.6	0	0.5	6.1
	5 mmol		0.32	0	54.8	0	0	0	0	0	0	0	0	3.2	0	0	0
	15 mmol		0.07	0.7	100	0	0	0	0	0	0	0	0	0	0	0	0
	25 mmol		0.67	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4: Cytogenetic analysis of *Vicia faba* 'Nobaria 3' root tip treated with three concentrations of AlCl₃ for three exposure times. Abbreviations: Mitotic index (MI), total abnormalities, interphase and mitotic phases (prophase, metaphase and ana-telophase), include normal (Total) and abnormal (Abn.) mitotic phases.

Treatment	Exposure time (h)	AlCl ₃ conc.	Total mitosis	MI (%)	% Total ab-normal	% Interphase			% Prophase			% Metaphase			% Ana-telophase		
						Total	Abn.	Abn.	Total	Abn.	Abn.	Total	Abn.	Abn.	Total	Abn.	Abn.
6	Control	355	7.10±0.36	4.09±0.42	92.9	0.4	35.9	0.4	23.6	1.4	40.5	0					
	5 mmol	91	1.87±0.30*	87.25±5.68*	97.6	2.4	65.7	50.6	27.4	27.3	6.9	4.10					
	15 mmol	49	1.98±0.12*	78.30±5.85*	96.7	1.4	67.9	20.7	11.3	9.4	20.8	13.32					
	25 mmol	130	2.60±0.30*	86.00±2.00*	97.4	1.6	41.9	20.3	23.2	18.4	34.9	17.7					
12	5 mmol	160	3.20±0.15*	92.77±2.15*	97.6	0.8	52.2	21.7	13.1	8.6	34.7	21.7					
	15 mmol	110	2.19±0.46*	86.87±0.95*	96.2	2.6	25.9	22.9	22.2	22.2	51.9	24.07					
	25 mmol	22	0.44±0.03*	95.70±3.55*	97.8	1.3	30.2	27.9	46.5	45.3	23.3	16.3					
24	5 mmol	30	0.60±0.20*	93.64±1.70*	98.3	0.7	17.4	17.4	43.5	39.1	39.1	30.4					
	15 mmol	27	0.54±0.04*	87.00±3.00*	97.5	1.8	42.9	28.5	32.1	28.5	25	34.3					
	25 mmol	16	0.33±0.08*	97.27±1.80*	99.5	1.7	0	0	3.1	5.2	96.9	63.2					

* The mean difference is significant at the 0.05 level.

Table 5: Types and percentage (%) of chromosomal abnormalities in *Vicia faba* 'Nobaria 3' root tip treated with three concentrations of AlCl₃ for three exposure times. Abbreviations: C-metaphase (C-m), sticky (Stick), star, break, disturbed (Dist), and diagonal (Diag), bridge, free, C-anaphase (C-ana), micronuclei (MN) and bi-nucleated cell (BN).

Treatment	Exposure time (h)	AlCl ₃ conc.	Interphase abnormalities					Metaphase abnormalities					Ana-telophase abnormalities				
			BN	C-m	Stick	Star	Break	Dist	Diag	Free	Bridge	Dist	Stick	Break	Star	C-ana	
6	Control	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0	0
	5 mmol	2.44	0.23	6.8	1.4	0	1.4	17.8	1.4	1.4	0	0	0	0	0	0	0
	15 mmol	0.91	0.05	0	0	1.9	0	7.5	1.9	0	5.7	1.9	0	5.7	0	0	0
	25 mmol	0.80	0.09	6.1	1.3	0	2.6	7.7	1.3	4	11.3	0.6	0	1.3	0.3	0.6	0.6
12	5 mmol	0.71	0.1	0	4.3	0	4.3	0	4.3	0	0	0	4.3	4.3	8.7	0	0
	15 mmol	1.38	0.1	0.9	0.9	0	1.9	18.5	10.2	0	7.4	0.9	0	3.7	1.8	0.9	0.9
	25 mmol	1.88	0.1	40.6	1.2	0	1.2	2.3	4.7	0	2.3	2.3	1.2	1.2	4.7	0	0
24	5 mmol	2.40	0.1	30.4	0	4.3	0	4.3	8.7	0	17.4	0	4.3	0	0	0	0
	15 mmol	1.46	0	10.7	0	3.6	7.1	7.1	3.6	0	10.7	0	0	0	0	0	0
	25 mmol	1.45	0.2	5.3	0	0	0	0	63.2	0	0	0	0	0	0	0	0

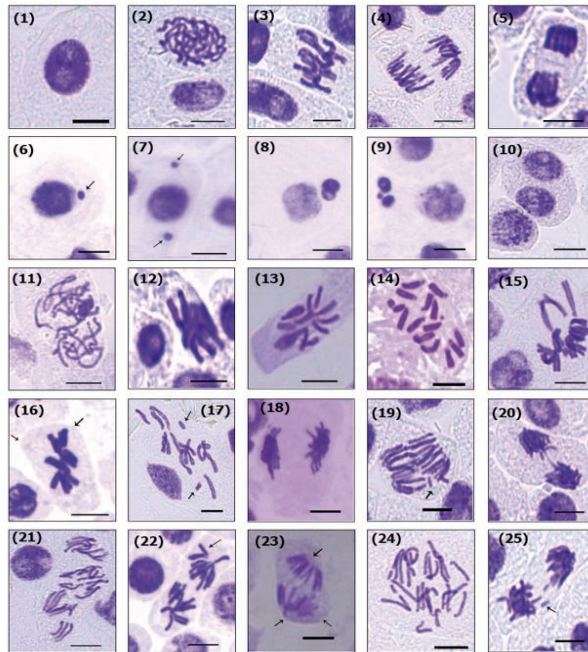


Figure 1: Chromosomal aberrations in *Vicia faba* 'Giza 843 and Nobaria 3' root tips under the influence of $AlCl_3$: interphase (1), prophase (2), metaphase (3), anaphase (4), telophase (5), micronuclei with different sizes and different number (6, 7, 8, 9), binucleated cell (10), irregular prophase (11), sticky metaphase (12), star metaphase (13), C-metaphase (14), dis 5rturbed metaphase (15), diagonal metaphase (16), metaphase break (17), sticky anaphase (18), anaphase break (19), anaphase bridge (20), C-anaphase (21), anaphase with free chromosome (22), diagonal anaphase (23), disturbed anaphase (24) and sticky anaphase break (25). The scale bar equals 10 μm .

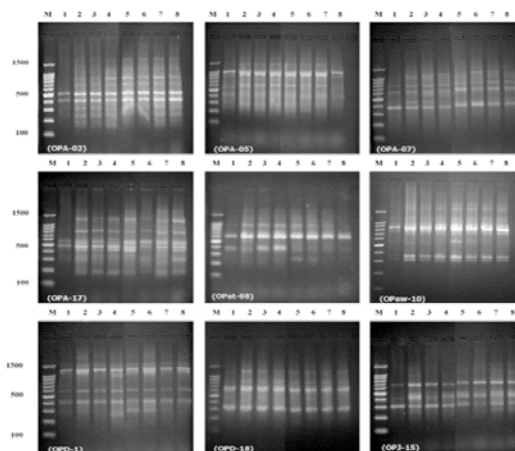


Figure 2: RAPD profile generated by 9 primers using roots of two *Vicia faba* cultivars treated with 5 mmol $AlCl_3$ for different periods. Lanes 1, 2, 3 and 4: 'Giza 843' for 0 (control), 6, 12 and 24 h, respectively. Lanes 5, 6, 7 and 8: 'Nobaria 3' for 0 (control) 6, 12 and 24 h, respectively. M: DNA ladder.

baria 3' cells were higher those of 'Giza 843'. In concern to ana-telophase abnormalities, the frequency of diagonal chromosomes under different $AlCl_3$ treatments was higher in 'Giza 843' than in 'Nobaria 3' (Tables 3 and 5). In comparison to other chromosomal abnormalities, free chromosomes appeared in low frequency in both culti-

vars (Table 3 and Table 5); it never appeared when low concentration of $AlCl_3$ in combination with short exposure time was applied.

Root tips treated with 5 mmol $AlCl_3$ for different periods were subjected to molecular analysis using RAPD (Table 6) and ISSR primers (Table 7). When ten RAPD

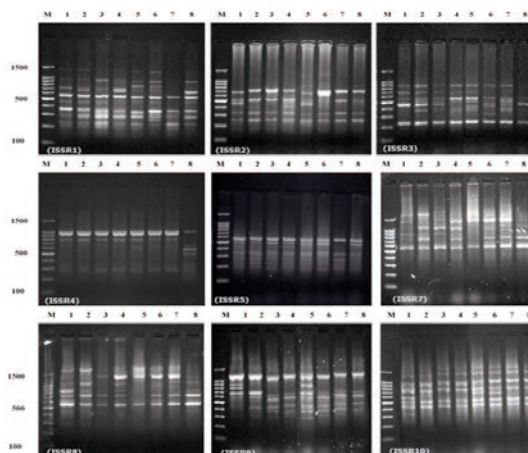


Figure 3: ISSR profile generated by 9 primers using roots of two *Vicia faba* cultivars treated with 5 mmol AlCl_3 for different periods. Lanes 1, 2, 3 and 4: 'Giza' 843 for 0 (control), 6, 12 and 24 h, respectively. Lanes 5, 6, 7 and 8: 'Nobaria 3' for 0 (control) 6, 12 and 24 h, respectively. M: DNA ladder.

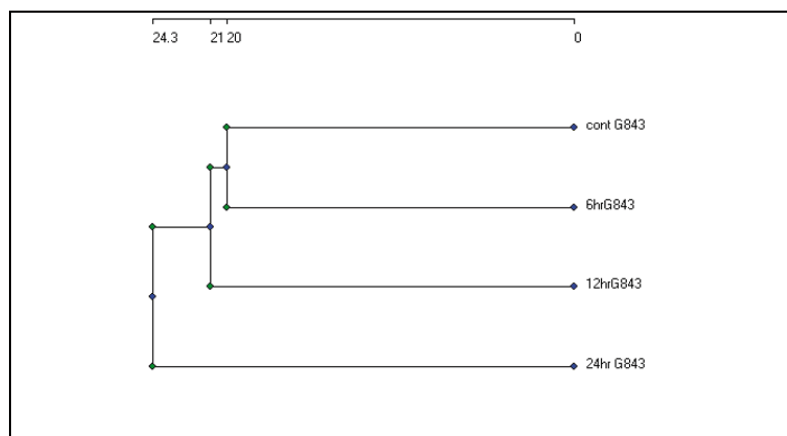


Figure 4: UPGMA based cluster tree of *Vicia faba* 'Giza 843' exposed to 5 mmol AlCl_3 for 6, 12 and 24 h based on ten RAPD and nine ISSR primers.

primers were used for amplification of genomic DNA of two faba bean cultivars ('Giza 843' and 'Nobaria 3'), different profiles were obtained (Table 6; Fig. 2). They expressed 27 and 26 polymorphic, 20 and 20 monomorphic, 8 and 10 unique out of total 55 and 56 bands when genomes of 'Giza 843' and 'Nobaria 3' were amplified, respectively. Consequently, while percentage of polymorphism ranged from 0 % (OPD-18) to 85.72 % (OPA-17) with an average of 61.74 % in 'Giza 843', they ranged from 20 % (OPD-18) to 90.91 % (OPA-17) with an average of 61.24 % in 'Nobaria 3'.

The applied ISSR primers on 'Giza 843' genome resulted in amplification of 38 polymorphic out of 69 fragments (55.07 %), they included 18 monomorphic, 9 unique and 38 polymorphic bands (Table 7; Fig. 3). Consequently, the percentage of polymorphism in 'Giza 843'

PCR products ranged from 25 % (ISSR5 or ISSR10) to 100 % (ISSR9) with an average of 65.36 %; it was higher than that of RAPD primers. When root tips of 'Nobaria 3' were subjected to AlCl_3 for different periods using ISSR primers, 72 fragments were amplified including 22 monomorphic, 7 unique and 43 polymorphic bands. Percentage of polymorphism ranged from 25 % (ISSR5) to 100 % (ISSR9) with an average of 71.59 %; it was higher than that of RAPD primers.

Cluster tree based on ten RAPD and nine ISSR primers of 'Giza 843' cultivar resulted in grouping of control plant with others subjected to 5 mmol Al for 6 or 12 h (Fig. 4). On the other side, the dendrogram of 'Nobaria 3' included two main clusters; the first cluster contained control plants and others subjected to AlCl_3 for 6 h. The

Table 6: Ten RAPD primers, annealing temperature, size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands and unique bands identified per primer used to access genome stability of two *Vicia faba* cultivars (Giza 843 and Nobarria 3) under the influence of Al stress.

Primer	Annealing temperature (°C)	Polymorphic bands		Monomorphic bands		Unique bands		Total bands		Size range (bp)	Polymorphism (%)
		Giza 843		Nobarria 3		Giza 843		Nobarria 3			
		Giza 843	Nobarria 3	Giza 843	Nobarria 3	Giza 843	Nobarria 3	Giza 843	Nobarria 3		
OPA-02	32	4	3	2	2	0	0	6	5	141-753	66.67
OPA-05	30	4	3	3	3	1	0	8	6	225-1102	62.50
OPA-07	30	3	2	2	2	1	3	6	7	497-1428	66.67
OPA-17	30	5	7	1	1	1	3	7	11	162-1811	85.72
OPat-08	32	2	1	1	1	0	1	3	3	218-958	66.67
OPaw-10	30	3	4	1	1	1	0	5	5	313-912	80.00
OPD-1	32	2	2	3	3	3	0	8	5	236-1364	62.5
OPD-18	30	0	0	4	4	0	1	4	5	292-1278	00.00
OPI-15	30	2	2	1	1	0	0	3	3	229-737	66.67
OPP-13	32	2	2	2	2	1	2	5	6	345-876	60.00
Total		27	26	20	20	8	10	55	56		61.74

Table 7: Nine ISSR primers, annealing temperature, size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands and unique bands identified per primer used to access genome stability of two *Vicia faba* cultivars (Giza 843 and Nobarria 3) under the influence of Al stress.

Primer	Annealing temperature (°C)	Polymorphic bands		Monomorphic bands		Unique bands		Total bands		Size range (bp)	Polymorphism (%)
		Giza 843		Nobarria 3		Giza 843		Nobarria 3			
		Giza 843	Nobarria 3	Giza 843	Nobarria 3	Giza 843	Nobarria 3	Giza 843	Nobarria 3		
ISSR1	56	6	7	3	3	1	1	10	11	175-1287	72.73
ISSR2	60	4	5	1	1	2	0	7	6	239-703	83.33
ISSR3	58	6	6	1	1	0	1	7	8	206-870	87.50
ISSR4	50	1	3	2	2	0	2	3	7	167-985	71.43
ISSR5	50	2	2	6	6	0	0	8	8	188-565	25.00
ISSR7	38	5	6	1	1	1	1	7	8	804-1376	87.50
ISSR8	36	5	5	2	2	2	0	9	7	295-1489	71.42
ISSR9	32	7	6	0	0	3	0	10	6	169-828	100
ISSR10	38	2	3	6	6	0	2	8	11	457-1557	45.45
Total		38	43	18	22	9	7	69	72		65.36

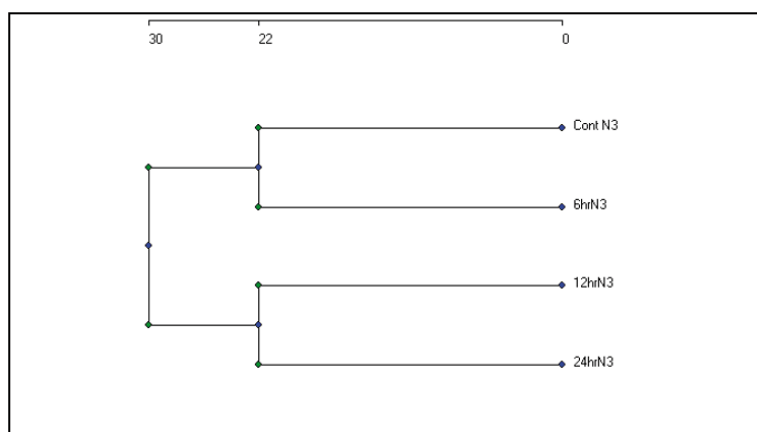


Figure 5: UPGMA based cluster tree of *Vicia faba* 'Nobaria 3' exposed to 5 mmol AlCl_3 for 6, 12 and 24 h based on ten RAPD and nine ISSR primers.

second cluster included plants subjected to AlCl_3 for 12 or 24 h (Fig. 5).

4 DISCUSSION

When seed were germinated and subjected to AlCl_3 concentrations, they showed retardation of seed germination and seedling growth, which were lower in 'Giza 843' than in 'Nobaria 3'. Retardation in plant growth under the influence of Al toxicity could be attributed to DNA damage (Achary and Panda, 2010), inhibition of cell division and elongation (De Campos and Viccini (2003), stimulation of oxidative stress (Rout et al., 2001), inhibition of water movement through roots, and retardation of root development and the ability of plants to develop symbiosis (Aroca et al., 2007; Belachew and Stoddard, 2017). Consequently, aluminum toxicity exacerbates the effects of other abiotic stresses (Muktadir et al., 2020). Seedlings were used in this study because they are more susceptible to AlCl_3 than older plants (Thawornwong and van Diest, 1974) especially when relatively high concentration of AlCl_3 was used. Aluminum toxicity on faba bean cultivars as well as other plant species was reported (Rout et al., 2001; Yi et al., 2010; Altway et al., 2016), but its effect varies within plant species and genotypes (Rengel, 1996). To mimic many unfertile acid soils around the globe, 50 mmol AlCl_3 for 72 h was used to test germplasm sensitivity to Al toxicity (Domingues et al., 2013). In our study, high concentrations of AlCl_3 up to 25 mmol were used for cytogenetic studies. Under these conditions, seedling growth of 'Giza 843' was less influenced by AlCl_3 than 'Nobaria 3'.

Aluminum chloride caused a significant decrease in mitotic activities of root tip cells of 'Giza 843' and 'No-

baria 3'. The reduction in MI of 'Giza 843' was lower than that of 'Nobaria 3' indicating that 'Giza 843' was more Al-tolerant than 'Nobaria 3'. The obtained data indicate that AlCl_3 induced cell cycle defects in faba bean root cells (Zhang et al., 2009; Yi et al., 2010). The lowest values of MI were detected when the highest concentration of AlCl_3 (25 mmol was used for 24 h leading to the complete inhibition of cell division in 'Giza 843'. Inhibition of mitotic cell cycle was attributed to the retardation of cell cycle during interphase (Mohandes and Grant, 1972), decline of energy resources (Rost and Morrison, 1984), inhibition of transition from G1 to S and G2 (Van't Hoff, 1985) and binding of Al on DNA molecules (Matsumoto et al., 1976).

While total mitosis and MI drastically decreased, total abnormalities increased in both cultivars when they were subjected to AlCl_3 for 6 or 12 h. Under these conditions, total abnormalities in 'Giza 843' were lower than those of 'Nobaria 3', which may be due to lower abnormalities in prophase and ana-telophase stages, especially under the influence of the lowest exposure period. Increased exposure time to AlCl_3 for more than 6 h resulted in irregular trends of the obtained data. Under Al stress, cell divisions in faba bean root tips was often associated with reduction in genomic template stability and an increase in RAPD band frequencies (Rout et al., 2001; Taspinar et al., 2018). Hartig and Beck (2006) confirmed the survival of the stressed cells if they tolerated the occurred mutation (Hartig and Beck, 2006). In both cultivars, while frequency of interphase was very high under the highest Al treatment (25 mmol for 24 h), the prophase was inhibited completely in this study and others (Yi et al., 2010).

The frequency of MN increased under the influence of all AlCl_3 treatments, this increase was lower in 'Giza

843' than 'Nobaria 3'. Micronuclei formation was used as an effective tool to measure of chromosomal DNA damages and analyzes the mutagenic effect of chemicals (Fenech, 2008). These studies indicated that some of the cells enter the mitotic cell division with damaged DNA (Hartig and Beck, 2006). Under stress conditions, positive correlation was detected between chromosomal abnormalities and MN, and antioxidant enzymes and lipid peroxidation (Souguir et al., 2011). Since 'Giza 843' had less micronuclei and chromosomal abnormalities than 'Nobaria 3' under $AlCl_3$ stress, 'Giza 843' was considered more tolerant to Al than 'Nobaria 3' cultivar.

While several types of chromosomal abnormalities at all stages of mitosis were detected under $AlCl_3$ treatments, they depended on the used cultivar. The most common types of these abnormalities were bridges, laggards, fragments and micronuclei as was reported by Yi et al. (2010). Under the influence of different $AlCl_3$ treatments, frequencies of appearance of different abnormalities including C-anaphase, star metaphase chromosomes, and breaks, sticky and disturbed chromosomes during metaphase or anaphase were higher in 'Nobaria 3' than those of 'Giza 843'. Our data confirmed that Al is a genotoxic and cytotoxic reagent in plant cells leading to chromosomal abnormalities in several forms (Yi et al., 2010).

Under the toxic effect of $AlCl_3$, changing the frequency of primers binding loci increased as cytogenetical events increased; which could be identified by the values of the obtained polymorphism. When ten of RAPD primers were used for amplification of genomic DNA of 'Giza 843' and 'Nobaria 3', the polymorphism average 61.74 % in 'Giza 843' and 61.24 % in 'Nobaria 3'. Link et al. (1995) confirmed that RAPD was useful molecular markers to study genetic variation in faba beans. In our work, application of RAPD technique to detect genetic variation due to Al treatments in faba bean was not recommended.

Application of ISSR primers on 'Giza 843' and 'Nobaria 3' genomes resulted in amplification of 69 and 72 fragments, respectively. ISSR technique was efficiently used for classifying faba bean and other plant species (Abdel-Razzak et al., 2012; Salem and Hassanein, 2017; Hassanein et al., 2018). The calculated polymorphism average of ISSR primers of 'Giza 843' was lower than that of 'Nobaria 3' indicating that 'Giza 843' genome was more stable than that of Nobaria 3 under Al stress. The detected DNA polymorphism may be due to mismatching at the primer site, appearance of a new primer site and/or change the distance between two opposite primers. These events may result from point, and chromosomal mutations/abnormalities (Kumar and Rai, 2006). Consequently, these events could be detected by cytogenetical

techniques (through micronuclei formation and chromosomal abnormalities) or molecular techniques such as ISSR (through polymorphism average). Data obtained from both techniques confirmed that Al tolerance of 'Giza 843' was higher than that of 'Nobaria 3'. ISSR was efficiently used to detect variation in faba bean (Abdel-Razzak et al., 2012). Combination of cytogenetical and molecular techniques for efficient evaluation of genetic variation in plants was recommended in this study and others (Begum and Alam, 2017; Hossain et al., 2017).

Construction of dendrograms depending on the data of molecular techniques and study of their details supplemented us with another evidence confirmed the harmony between cytogenetical and molecular biology techniques as well as seedling growth parameters. The obtained cluster tree of 'Giza 843' grouped of control plant with others subjected to 5 mmol Al for 6 or 12 hr. On the other side, the cluster tree of 'Nobaria 3' grouped control plants with only others subjected to 5 mmol $AlCl_3$ for 6 hr. Comparison between the two obtained dendrograms, polymorphism and cytogenetical data indicated that 'Giza 843' tolerated Al more than 'Nobaria 3'.

5 REFERENCES

- Abdelhamid M. T., Shokr M. M. B. & Bekheta M. A. (2010). Growth, root characteristics, and leaf nutrients accumulation of four faba bean (*Vicia faba* L.) cultivars differing in their broomrape tolerance and the soil properties in relation to salinity. *Communication Soil Science of Plant Analysis*, 41, 2713–2728. <https://doi.org/10.1080/00103624.2010.518263>
- Abdel-Razzak H. S., Alfrmawy A. M., Ibrahim H. M. & El-Hanafy A. A. (2012). Genetic diversity in faba bean (*Vicia faba* L.) using Inter-Simple Sequence Repeat (ISSR) markers and protein analysis. *Life Science Journal*, 9, 497-503.
- Achary V. M. M. & Panda B. B. (2010). Aluminium-induced DNA damage and adaptive response to genotoxic stress in plant cells are mediated through reactive oxygen intermediates. *Mutagenesis*, 25(2), 201-209. <https://doi.org/10.1093/mutage/geb063>
- Altwayt N. H., El-Sayed O. E., Aly N. A. H., Baeshen M. N. & Baeshen N. A. (2016). Molecular and cytogenetic assessment of *Dipterygium glaucum* genotoxicity. *Anais da Academia Brasileira de Ciências*, 88, 623–634. <https://doi.org/10.1590/0001-3765201620150208>
- Aroca, R.; Porcel, R. & Ruiz-Lozano, J. M. (2007). How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytologist*, 173, 808–816. <https://doi.org/10.1111/j.1469-8137.2006.01961.x>
- Begum K. N. & Alam S. S. (2017). Karyotype, RAPD and ISSR analysis of four specimens in *Gynura nepalensis* DC. *Cy-*

- tologia*, 82(4), 423–428. <https://doi.org/10.1508/cytologia.82.423>
- Belachew, K.Y. & Stoddard, F. L. (2017). Screening of faba bean (*Vicia faba* L.) accessions to acidity and aluminium stresses. *PeerJ*, 5, e2963. <https://doi.org/10.7717/peerj.2963>
- Bennet R. J. & Breen CM (1991). The aluminium signal: New dimensions to mechanisms of aluminium tolerance. *Plant Soil*, 134, 153–166. <https://doi.org/10.1007/BF00010728>
- Darlington C. D. & La Cour L. F. (1976). *The handling of chromosomes*, 6th ed. George Allen and Unwin Ltd., London.
- De Campos J. M. S. & Viccini L. F. (2003) Cytotoxicity of aluminium on meristematic cells of *Zea mays* and *Allium cepa*. *Caryologia*, 56, 65–73. <https://doi.org/10.1080/00087114.2003.10589309>
- Domingues A. M., da Silva F., Freitas G., Ganança G. F., Nóbrega H., Slaski J. J. & de Carvalho M. A. P. (2013). Aluminium tolerance in bean traditional cultivars from Madeira. *Revista de Ciências Agrárias*, 36(2), 148–156.
- Duc G, Bao SY, Baum M & Redden B. (2010). Diversity maintenance and use of *Vicia faba* L. genetic resources. *Field Crops Research*, 115, 270–278. <https://doi.org/10.1016/j.fcr.2008.10.003>
- Fenech M. (2008). The micronucleus assay determination of chromosomal level DNA damage. *Methods of Molecular Biology*, 410, 185–216. https://doi.org/10.1007/978-1-59745-548-0_12
- Gopalan, H.N.B. 1999. Ecosystem health and human wellbeing: the mission of the International Program on Plant Bioassays. *Mutation Research*, 426, 99–102. [https://doi.org/10.1016/S0027-5107\(99\)00048-2](https://doi.org/10.1016/S0027-5107(99)00048-2)
- Hartig K. & Beck E. (2006). Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle. *Plant Biology*, 8, 389–396. <https://doi.org/10.1055/s-2006-923797>
- Hassanein A. M., Salem J. M., Faheed F. A. & El-nagish A. (2018). Effect of anti-ethylene compounds on isoenzyme patterns and genome stability during long term culture of *Moringa oleifera*. *Plant Cell Tissue Organ Culture*, 132, 201–212. <https://doi.org/10.1007/s11240-017-1326-0>
- Hossain, A., Afroz, M., Sultana, S. S. & Alam S.S. (2017). Karyotype and RAPD Diversity in Four Varieties of *Gossypium hirsutum* L. *Cytologia*, 82(5), 535–541. <https://doi.org/10.1508/cytologia.82.535>
- Kanaya N. 1., Gill B. S., Grover I. S., Murin A., Osiecka R., Sandhu S. S. & Andersson H. C. (1994). *Vicia faba* chromosomal aberration assay. *Mutation Research*, 310(2), 231–247. [https://doi.org/10.1016/0027-5107\(94\)90116-3](https://doi.org/10.1016/0027-5107(94)90116-3)
- Karimaei M. & Poozesh V. (2016). Effects of aluminum toxicity on plant height, total chlorophyll (Chl a+b), potassium and calcium contents in spinach (*Spinacia oleracea* L.). *International Journal of Farming and Allied Sciences*, 5(2), 76–82.
- Kotelnikova A., Fastovets I., Rogova O., Volkov D. S. & Stolbova V. (2019). Toxicity assay of lanthanum and cerium in solutions and soil. *Ecotoxicology and Environmental Safety*, 167, 20–28. <https://doi.org/10.1016/j.ecoenv.2018.09.117>
- Kumar G. & Rai P. (2006). Partial genome elimination through micronuclei in soybean (*Glycine max*). *National Academy Science Letters*, 29, 417–421.
- Legendre L. & Legendre P. (1983). *Numerical ecology: developments in environmental modelling*. Amsterdam, The Netherlands, pp 419. https://doi.org/10.1007/978-3-642-69024-2_56
- Link W., Dextrins C., 3. Elsevier,
- Singh M., Schwall M. & Melschimmer A. E. (1995). Genetic diversity in European and Mediterranean faba beans germplasm revealed by RAPD markers. *Theoretical and Applied Genetics*, 90, 27–32. <https://doi.org/10.1007/BF00220992>
- Llugany M., Poschenrieder C. & Barcelo J. (1995). Monitoring of aluminium-induced inhibition of root elongation in four maize cultivars differing in tolerance to aluminium and proton toxicity. *Physiologia Plantarum*, 93, 265–271. <https://doi.org/10.1111/j.1399-3054.1995.tb02227.x>
- Matsumoto H., Hirasawa E., Torikai H. & Takahashi E. (1976). Localization of absorbed Al in pea root and its binding to nucleic acids. *Plant and Cell Physiology*, 17(1), 127–137. <https://doi.org/10.1093/oxfordjournals.pcp.a075252>
- May H. M. & Nordstrom D. K. (1991). Assessing the solubilities and reaction kinetics of aluminous minerals in soils. In: *Soil acidity*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 125–148. https://doi.org/10.1007/978-3-642-74442-6_6
- Mohandas T. & Grant W. F. (1972). Cytogenetic effects of 2,4-d and amitrole in relation to nuclear volume and DNA content in some higher plants. *Canadian Journal of Genetics and Cytology*, 14(4), 773–783. <https://doi.org/10.1139/g72-095>
- Mohanty S., Das A., Das P. & Mohanty P. (2004). Effect of a low dose of aluminum on mitotic and meiotic activity, 4C DNA content, and pollen sterility in rice, *Oryza sativa* L. cv. Lalat. *Ecotoxicology and Environmental Safety*, 59, 70–75. <https://doi.org/10.1016/j.ecoenv.2003.07.017>
- Muktadir M. A., Adhikari K. N., Merchant A., Belachew K.Y., Vandenberg A., Stoddard F. L. & Khazaei H. (2020). Physiological and Biochemical Basis of Faba Bean Breeding for Drought Adaptation. *Agronomy*, 10, 1345; <https://doi.org/10.3390/agronomy10091345>
- Porebski S., Bailey L. G. & Baum B. R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reports*, 15, 8–15. <https://doi.org/10.1007/BF02772108>
- Rengel Z. (1996). Uptake of aluminium by plant cells. *New Phytologist*, 134, 389–406. <https://doi.org/10.1111/j.1469-8137.1996.tb04356.x>
- Richard S. & Peter H. (2007). Community Analysis Package 4.0. *Searching for structure in community data*. Printed in Lymington, UK.
- Rost T. L. & Morrison S. L. (1984). The comparative cell cycle and metabolic effects of chemical treatments on root tips meristems. II. Protham, chloroprotham and 2,4-dinitrophenol. *Cytologia*, 49, 61–72. <https://doi.org/10.1508/cytologia.49.61>
- Rout G. R., Samantaray S. & Das P. (2001). Aluminium toxicity in plants: a review. *Agronomie*, 21, 3–21. <https://doi.org/10.1051/agro:2001105>
- Ryan P., Ditomaso J. M. & Kochian V. (1993). Aluminium toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. *Journal of Experimental Botany*, 44 (2), 437–446. <https://doi.org/10.1093/jxb/44.2.437>
- Salem J. & Hassanein A. M. (2017). In vitro propagation, mi-

- crotuberization, and molecular characterization of three potato cultivars. *Biologia Plantarum*, 61, 427–437. <https://doi.org/10.1007/s10535-017-0715-x>
- Sivaguru M. & Horst W. J. (1998). The distal part of the transition zone is the most aluminum-sensitive apical root zone of maize. *Plant Physiology*, 116, 155–163. <https://doi.org/10.1104/pp.116.1.155>
- Souguir D, Ferjani E, Ledoigt G, Goupil P (2011) Sequential effects of cadmium on genotoxicity and lipoperoxidation in *Vicia faba* roots. *Ecotoxicology*, 20, 329–336. <https://doi.org/10.1007/s10646-010-0582-0>
- Suso M. J., Gilsanz S., Duc G., Marget P. & Moreno M. T. (2006). Germplasm management of faba bean (*Vicia faba* L.): Monitoring intercrossing between accessions with inter-plot barriers. *Genetic Resources and Crop Evolution*, 53, 1427–1437. <https://doi.org/10.1007/s10722-005-6844-7>
- Taspinar M. S., Aydin M., Sigmaz B., Yagci S., Arslan E. & Guleray Agar G. (2018). Aluminum-Induced Changes on DNA Damage, DNA Methylation and LTR Retrotransposon Polymorphism in Maize. *Arab Journal of Science and Engineering*. 43:123–131. <https://doi.org/10.1007/s13369-017-2697-6>
- Thawornwong N. & Van Diest A. (1974). Influences of high acidity and aluminum on the growth of lowland rice. *Plant and Soil*, 41 (1), 141–159. <https://doi.org/10.1007/BF00017951>
- Van't Hoff J. (1985). Control points within cell cycle. In: *The cell division cycle in plants*. Edited by: J.A. Bryant and Francis, D. SEB Seminar series 26. Cambridge University Press. Pp 1–13.
- Yi M., Yi H., Li H. & Wu L. (2010). Aluminum induces chromosome aberrations, micronuclei, and cell cycle dysfunction in root cells of *Vicia faba*. *Environmental Toxicology*, 25, 124–129. <https://doi.org/10.1002/tox.20482>
- Zhang H. M., Zhang S. S., Meng Q. M., Zou J., Jiang W. S. & Liu D. H. (2009.) Effects of aluminum on nucleoli in root tip cells, root growth and the antioxidant defense system in *Vicia faba* L. *ACTA BIOLOGICA CRACoviENSIA Series Botanica*, 51(2), 99–10.