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# ASSESSMENT OF GENETIC VARIATIONS IN EMS-EXPOSED *PETUNIA* TESTED FOR SALT *IN VITRO* TOLERANCE USING RAPD*

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Abstract

Random Amplified Polymorphic DNA (RAPD) was used to assess the genetic variability within somaclones of *Petunia* cv. Prism Red exposed to 0.5 mM ethyl methane-sulphonate (EMS), and tested for salt tolerance *in vitro*. Twenty RAPD primers were utilized; 8 out of which amplified specific fragments generating a total of 39 alleles, with a mean of 3.9 alleles per locus. Three arbitrary oligonucleotide primers revealed polymorphisms between non-mutated and mutated plants. Whereas, six RAPD primers generated polymorphic products characteristic only for EMS putative mutants or for EMS putative mutants tested for salt tolerance. The extent of polymorphism indicated the existence of variability within DNA in induced mutated somaclones. Cluster analysis using the Nei's similarity coefficient values and UPGMA algorithm detected genetic variation within non-mutated and mutated plants, as they are placed in different clusters/groups far from each other. Similarity matrices mostly ranged from 0.5 to 0.99. Results were indicative that induced mutation bears great potential in improving petunia for salinity resistance which can be considered as potential samples for further breeding programs.

## Introduction

One of the main aspects of modern plant breeding is increasing the crop tolerance to environmental stresses, effective use of water potential, resistance to pathogens, as well as the production of high-value bioactive compounds (PENNA and JAIN 2017). One of the major environmental stress

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factors, which limits the growth and development of many important plant species, is salinity. This problem has occurred mainly in the southern countries of Europe (Hungary, Romania, Greece, Italy, and Spain), and reaches from 1 to 3 million ha (DALIAKOPOULOS et al. 2016). In northern Europe, salt is mostly used as a deicing agent for road maintenance and salinization occurs locally. In Poland, saline soil covers approx 5400 ha (DALIAKOPOULOS et al. 2016, KUJAWSKA et al. 2020). Ornamental and floriculture species have high horticultural and esthetic value, therefore it is very important to determine the salt tolerance of popular bedding plants during greenhouse production. The most effective breeding program means that can deal with soil salinity is to grow cultivars that can establish and be productive on such soil (NIU and RODRIGUEZ 2006, BIDABADI et al. 2011, VILLARINO and MATTSON 2011). Salt tolerance of bedding plants is highly variable, depending on the species or even cultivar, climatic conditions, and irrigating methods (NIU and RODRIGUEZ 2006). Development of plants tolerance to salinity in many crops have been reported about a wide range of plant species including sunflower (SADAK et al. 2010), tomato (SHALABY and EL-BANNA 2013, KRUPA-MAŁKIEWICZ and KULPA 2018), Musa (MIRI et al. 2014), petunia (KRUPA-MAŁKIEWICZ and FORNAL 2018).

Belonging to the *Solanaceae* petunia (*Petunia* × *atkinsiana* D. Don) is being one of the most popular bedding flowers worldwide. May serve as a comparative genetic and molecular biology model plant in the exploration of the molecular origin of some of the developmental diversity of important traits. Moreover, petunia has a short lifecycle, easy culture conditions, easy propagation (both sexual and asexual), biochemical analysis, and the development of stable and the plant is amenable to molecular analysis (DARQUI et al. 2017). The progress in the genetic improvement of horticultural and ornamental plants is slow, and hence greater efforts are required to induce mutations (BERENSCHOT et al. 2008, XU et al. 2009, VILLARINO and MATTSON 2011).

Breeding via mutation has been used to obtain genotypes with increased tolerance to environmental stress by BHAGWAT and DUNCAN (1998), JAIN (2000), BAIRU et al. (2011), MIRI et al. (2014), GADAKH et al. (2017), KRUPA-MAŁKIEWICZ et al. (2017), and ABDULHADI et al. (2019). As a result, the obtained putative mutants can have several desirable traits, e.g. disease resistance, high yield, quality, plant architecture, and abiotic stress tolerance (ASLAM et al. 2017, GADAKH et al. 2017, PENNA and JAIN 2017, GERAMI et al. 2019). Interestingly emerging traits in mutated populations can be identified by various biochemical or physiological methods (PENNA and JAIN 2017). However, several putative mutants derived by this method

can have different phenotypic effects, and often a majority of them are discarded possibly due to the lack of appropriate phenotypic (NADEAU 2000, LESTARI 2016, SRIVASTAVA et al. 2018). The use of molecular markers such as randomly amplified polymorphic DNA (RAPD) allow studying the level of diversity and to establish an index of genetic similarity among obtained variants (ABDULHADI et al. 2019). The current study proved that the RAPD technique was efficient in detecting genetic variation and thus can be used in plant breeding programs. Genetic variability using this technique has been studied by BHATTACHARYA et al. (2010) in *Cymbopogon winterianus*; ASLAM et al. (2017) in *Capsicum annuum* L., GADAKH et al. (2017) in sugarcane; and SRIVASTAVA et al. (2018) in orchid.

Therefore, in the present study RAPD technique was used to evaluate the genetic diversity among *Petunia* × *atkinsiana* D. Don cv. Prism Red plants, obtained from callus cultures treated with ethyl methane-sulphonate (EMS) and selected for salt tolerance *in vitro*. This study also helps to identify RAPD markers that would differentiate salinity resistance from susceptible clones.

## Materials and Methods

### Plant Material and Explant Source

Plants used in this study were obtained by KRUPA-MAŁKIEWICZ et al. (2017) in an experiment aimed to establish a protocol for produce petunia somaclones that are tolerant of salinity stress using *in vitro* EMS mutagenesis and *in vitro* selection method. Details on the protocol of explants, media preparation, culture conditions, and experimental design, as well as regeneration results were described by KRUPA-MAŁKIEWICZ et al. (2017). Clones were obtained from callus culture, initiated from the leaves. Callus was treated by 0.5 mM EMS and screening for salt tolerance using 50, 100, and 150 mM NaCl *in vitro*. Since the present experiment corresponds to the previous one performed by KRUPA-MAŁKIEWICZ et al. (2017), details of the morphological characteristics obtained are shown in Table 1 and Table 2.

Leaves of *Petunia* × *atkinsiana* D. Don cv. Prism Red were plant material. In the genetic analysis using RAPD techniques, to search for differentiating polymorphisms, twenty-five somaclones (Table 2) were selected, including a non-mutated clone as a control (sample No. 1), six clones mutated with 0.5 mM EMS (No. 2 to 7) and eighteen mutated clones tested for salinity (No. 8 to 25). Only DNA fragments that differentiated between the control sample and putative mutants' samples were considered mutation-

-linked markers. Morphological characterization of obtained somaclones was carried out based on six morphological traits (leaf habit, dwarfism, leaf pigmentation, the shape of the leaf, chimerism, and pigmentation of steam) compared to the control (Table 2) (SINK 1984).

Table 1

Somalones of *Petunia* × *atkinsiana* D. Don cv. Prism Red used in the study

| Number | Treatment | |
|---|---|---|
| | mutagenic treatment 0.5 mm EMS[a] | salinity[b] [mm] |
| 1 | 0 | 0 |
| 2–7 | 0.5 | 0 |
| 8–13 | 0.5 | 50 |
| 14–19 | 0.5 | 100 |
| 20–25 | 0.5 | 150 |

[a] – clones of petunia treated with 0.5 mM EMS and then screened under salinity
[b] – salt concentration that each clone was able to tolerate during four weeks

Table 2

Morphological characterization of putative mutants of *Petunia* × *atkinsiana* D. Don cv. Prism Red

| No. | Trait | Classification types | |
|---|---|---|---|
| | | non-mutated | mutated |
| 1 | leaf habit | erect | erect (74.2)*, dropping (25.8) |
| 2 | dwarfism | normal | normal (49.2), dwarf type (50.8) |
| 3 | leaf pigmentation | normal | normal (40.5), chlorosis (59.5) |
| 4 | shape of leaf | both sides rounded | both sides rounded (64.3), both sides pointed (35.7) |
| 5 | chimerism | absent | absent (68.1), chimerism (31.9) |
| 6 | pigmentation of steam | green | green (88.3), light green (7.2), yellow (4.5) |

* Percentage of classification is mentioned in parenthesis

## DNA Extraction and Quantification

For DNA extraction, approximately 20 mg lyophilized tissue of putative mutants of petunia and their corresponding mother plant, as a control, were isolated separately using the standard protocol of DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. The quality and quantity of DNA samples were measured by the spectrophotometer Epoch (BioTek) by the spectroscopy method. Only samples of high quality were used for RAPD-PCR.

## RAPD Amplification

Genetic diversity analysis was performed using RAPD PCR (*Random Amplification of Polymorphic DNA Polymerase Chain Reaction*) technique (WILLIAMS et al. 1990).

PCR amplification was carried out with a set of 20 oligonucleotide primers (Genomed and Biomers), selected for the analysis based on the references on the genetic diversity estimation in petunia (KRUPA-MAŁKIEWICZ and BIENIAS 2018). PCR reaction for RAPD was carried out in a reaction volume of 15 µl containing 10 × PCR buffer with $(NH_4)_2SO_4$, 2.0 mM of dNTPs, 2.0 mM $MgCl_2$, 0.5 µM of primer, 1.0 U *Taq* DNA polymerase (Thermo Scientific), and ~10 ng/µl of template DNA, according to the protocol described by WILLIAMS et al. (1990). Amplifications were carried out in DNA Engine Dyad® Thermalcycler (Biorad), according to the thermal program: initial denaturation step at 94°C for 3 min, 10 cycles – denaturation 94°C 1 min, annealing – 37°C at 30 s, elongation 72°C 30 s, 35 cycles – 94°C at 1 min, annealing 37°C at 30 s, elongation 72°C 1 min with the final extension step at 72°C 5 min.

## Electrophoresis

PCR products were mixed with 6 × Loading Dye Solution and were analyzed by electrophoresis on 1.5% agarose gel (Basica LE-Prona) at 8 V/cm for 110 min (in 1 × TBE buffer). Gene Ruler 100 bp DNA Ladder (Fermentas) was used as a size marker. PCR products were stained with ethidium bromide (0.1 mg $L^{-1}$) under UV light in a Syngene C:Box using GeneSnap Software. Amplification product profiles were scored for the presence (1) or absence (0) of bands, each of which was treated as independent characters regardless of their intensity.

## Data Analysis

The profiles generated in *Petunia* genotypes were analyzed to compute polymorphic information content (PIC) values according to ROLDÁN-RUIZ et al. (2000). Data analyses were conducted using the PhylTool software (BUNTJER 2001). The similarity matrix for RAPD primers was constructed using Nei's similarity coefficient values to find genotypic relationships (NEI and LI 1979). The generated matrix of similarities was analyzed by the unweighted pair-group method with arithmetic averages (UPGMA). The strength of the internal branches from the resulting tree was tested with TREECON bootstrap analysis application using 2,000 resamplings (VAN DE PEER and DE WACHTER 1994).

## Results and Discussion

Genetic variation in mutants can be induced either by physical and chemical mutagens or by specific tissue culture conditions. Many studies (MILER and JĘDRZEJCZYK 2018, ABDULHADI et al. 2019) have reported that the *in vitro* culture alone or combined with mutagenesis can be utilized to generate plants with increased genetic variability and mutants as a potential source of new commercial cultivars (BAIRU 2011, MIRI et al. 2014, LESTARI 2016, ASLAM et al. 2017, GADAKH 2017, MILER and JĘDRZEJCZYK 2018, SRIVASTAVA et al. 2018). Detection of variance is important in the subsequent use of these lines in crop improvement. However, the determination of the range of variability based on the observation of morphological features is difficult. Besides, the basis of the obtained changes may be genetic or epigenetic. Morphological traits are the product of gene and environmental interaction. Therefore obtained phenotypes do not determine the actual level of genetic variation. Among the different molecular techniques, RAPD is widely used to study the variation at DNA level among crops such as sugarcane (YADAV et al. 2006, GADAKH et al. 2017), *Capsicum annuum* L. (ASLAM et al. 2017), *Chrysanthemum morifolium* (MILER and JĘDRZEJCZYK 2018), orchid (SRIVASTAVA et al. 2018). Moreover, it has been indicated in the published studies dealing with the subject that RAPD markers are easy to use, cheap, and require no previous sequence information (SRIVASTAVA et al. 2018).

The genetic variability created in *Petunia* × *atkinsiana* D. Don cv. Prism Red through *in vitro* mutagenesis was efficiently assessed with molecular marker technique (RAPD). Genetic analysis in the characterization of petunia and its somaclones (putative mutants) allows detecting changes that occurred during *in vitro* culture and mutagen treatment. In this study, twenty selected oligonucleotide primers, which amplified stable and reproducible PCR products, were screened. Of these, 12 (pr4, pr176, pr269, pr44, pr66, pr88, pr199, pr107, pr139, pr144, pr920, pr1049) amplified monomorphic products did not confirm the variation within the tested samples. This may indicate the similarity of selected plants to the control plants. This may be due that most morphological variations were caused by a somatic or epigenetic mutation and might be distributed in the non-coding region of the genome.

From the eight RAPD primers (pr815, pr99, pr797, pr 29, pr519, pr447, pr875, pr598), which amplified polymorphic products, 39 alleles were detected, with a mean of 3.9 alleles per locus (Table 3). The size of the polymorphic products varied from 320 bp to 2100 bp. The number of alleles per locus ranged from 1 (pr66) to 8 (pr875). The mean polymorphism percentage was 29.8%, ranging from 20% to 50%. The PIC values for the RAPD loci ranged from 0.07 to 0.92, with an average of 0.66 (Table 3).

Table 3
Polymorphism information of eight primers responded during RAPD analysis of EMS induced mutants of *Petunia* × *atkinsiana* D. Don cv. Prism Red

| No. | Primer code | Number of alleles | Polymorphic alleles | Polymorphism [%] | PIC* values |
|---|---|---|---|---|---|
| 1 | pr815 | 4 | 1 | 25 | 0.92 |
| 2 | pr99 | 4 | 1 | 25 | 0.07 |
| 3 | pr797 | 4 | 1 | 25 | 0.92 |
| 4 | pr29 | 4 | 1 | 25 | 0.48 |
| 5 | pr519 | 5 | 1 | 20 | 0.92 |
| 6 | pr447 | 4 | 3 | 50 | 0.92 |
| 7 | pr875 | 8 | 2 | 25 | 0.28 |
| 8 | pr598 | 7 | 3 | 43 | 0.77 |

* polymorphic information content (PIC)

The polymorphism obtained in electropherograms confirmed the two types of segregation obtained in earlier studies (KRUPA-MAŁKIEWICZ and BIENIAS 2018). The first type of polymorphism, manifested by the absence or presence of a band in control or putative mutants, was observed on electropherograms of three primers pr815, pr99, pr875$_{[500\ bp]}$ (Fig. 1).

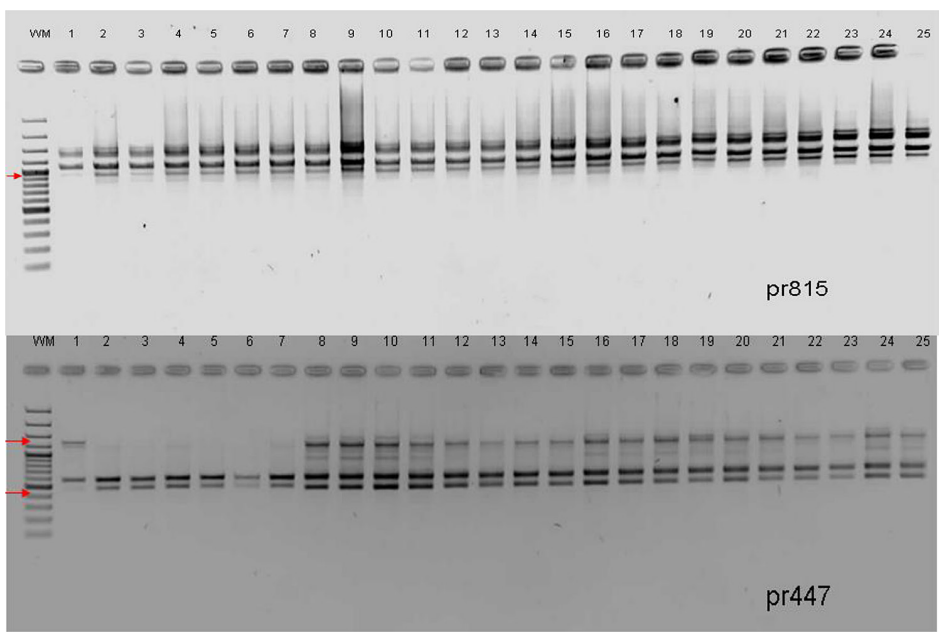



Fig. 1. RAPD profiles generated by primer pr815 generated polymorphic bands characteristic only for EMS putative mutants (2–7) and for EMS putative mutants of *Petunia* tested for salt tolerance (8–25), RAPD profiles generated by primer pr447 representing the absence of a band (490 bp) in control (1) and absence of a band (1250 bp) in putative mutants (2–7) of *Petunia* and arrows show different amplification in each regenerated variant, WM – weight mass DNA Gene Ruler; lines 1–25 see Table 1

The primers pr447, pr29, pr519, pr797, pr875$_{[400\ bp]}$, and pr598 generated polymorphic bands that are characteristic only for EMS putative mutants or for EMS putative mutants tested for salt tolerance (Fig. 1). To ensure that the occurrence of null alleles was not a failure of reaction, the assays were repeated twice. The results showed that the reproducibility was 90–100% under the same amplification conditions.

The results from the present study confirm with the study by YADAV et al. (2006) and GADAKH et al. (2017) among the *in vitro* mutagenized and selected for salt and drought stress sugarcane plants using RAPD marker technique. They screened sixty random decamer primers from which nine and ten gave sufficient intense bands, respectively. The extent of polymorphism, within the mutant clones, indicated the existence of considerable variation at the DNA level. MIRI et al. (2014) tested eleven RAPD primers to assess polymorphism in 22 banana clones. They found that only five amplified products resulted in polymorphic bands. Moreover, the PIC values for the microsatellite loci ranged from 0.25 to 0.85 with an average of 0.56. According to the above-mentioned authors, DNA markers showing an average PIC value of $> 0.5$ confirms the markers as highly informative.

Determining true genetic similarity between individuals using molecular markers is an important and decisive point for clustering which provides a visual idea about similarity presented in studied genotypes. In the presented study, the RAPD markers produced by eight primers were used to construct a similarity matrix (Table 4). A simple matching coefficient, ranging from 0.50 to 0.98, suggested a rather high similarity between the non-mutated petunia explants and its somaclones. The neighbor-joining method generated a dendrogram showing three main clusters of non-mutated explant and 24 putative mutants (Fig. 2). The first cluster induced only the control (nonmutated explant), while the second one contained the somaclones (2–7) that are only characteristic of EMS putative mutants. The third cluster included putative EMS mutant tested for salt tolerance (8–25). Figure 2 shows that the shortest genetic distances (the highest similarity value) were observed between the nonmutated explant and putative EMS mutants.

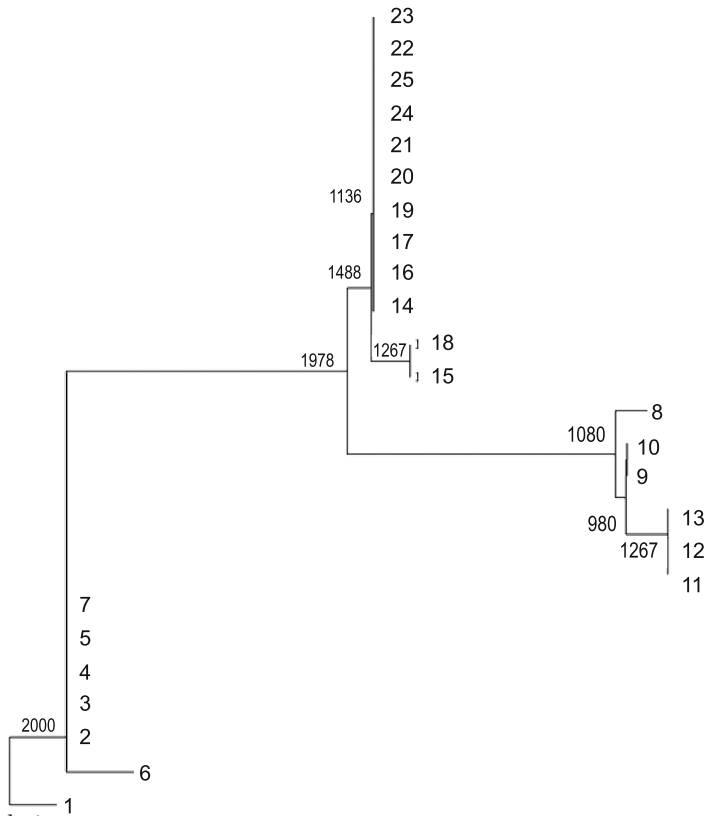
This may suggest that the EMS putative mutants did not accumulate many variations as compared to putative mutants tested for salt tolerance. The results obtained in this study confirm the study of other authors. For example, SHALABY and EL-BANNA (2013) evaluated the genetic similarity, which ranged from 0.82 to 0.99, among putative tomato mutants generated by EMS treatments; using RAPD and simple sequence repeat (SSR) markers. MIRI et al. (2014) reported that banana plants irradiated with gamma rays revealed variations among the clones, using RAPD and SSR markers.

Table 4

The simple matching coefficient of similarity matrix for *Petunia* × *atkinsiana* D. Don cv. Prism Red and its putative mutants determined from RAPD analysis by UPGMA cluster analysis

| | 1* | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.00 | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 0.92 | 1.00 | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 0.92 | 0.99 | 1.00 | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 0.93 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | | | | | | | | | | |
| 5 | 0.92 | 0.98 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | | | | | | | | | |
| 6 | 0.86 | 0.94 | 0.94 | 0.95 | 0.94 | 1.00 | | | | | | | | | | | | | | | | | | | |
| 7 | 0.92 | 0.98 | 0.98 | 0.98 | 0.98 | 0.94 | 1.00 | | | | | | | | | | | | | | | | | | |
| 8 | 0.55 | 0.63 | 0.63 | 0.63 | 0.63 | 0.63 | 0.63 | 1.00 | | | | | | | | | | | | | | | | | |
| 9 | 0.52 | 0.60 | 0.60 | 0.60 | 0.60 | 0.60 | 0.60 | 0.98 | 1.00 | | | | | | | | | | | | | | | | |
| 10 | 0.52 | 0.60 | 0.60 | 0.60 | 0.60 | 0.60 | 0.60 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | | | | |
| 11 | 0.50 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.94 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | | | |
| 12 | 0.50 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.94 | 0.98 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | | |
| 13 | 0.71 | 0.57 | 0.78 | 0.57 | 0.57 | 0.57 | 0.57 | 0.94 | 0.98 | 0.97 | 0.98 | 0.98 | 1.00 | | | | | | | | | | | | |
| 14 | 0.68 | 0.78 | 0.76 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 1.00 | | | | | | | | | | | |
| 15 | 0.71 | 0.76 | 0.78 | 0.76 | 0.76 | 0.71 | 0.76 | 0.76 | 0.78 | 0.78 | 0.81 | 0.81 | 0.81 | 0.97 | 1.00 | | | | | | | | | | |
| 16 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 1.00 | | | | | | | | | |
| 17 | 0.68 | 0.78 | 0.76 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 1.00 | | | | | | | | |
| 18 | 0.71 | 0.76 | 0.78 | 0.76 | 0.76 | 0.71 | 0.76 | 0.76 | 0.78 | 0.78 | 0.81 | 0.81 | 0.81 | 0.97 | 0.98 | 0.98 | 0.98 | 1.00 | | | | | | | |
| 19 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.99 | 0.98 | 1.00 | | | | | | |
| 20 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.97 | 0.97 | 0.98 | 1.00 | | | | | |
| 21 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.96 | 0.99 | 0.99 | 0.98 | 1.00 | | | | |
| 22 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.97 | 0.97 | 0.97 | 0.97 | 0.98 | 1.00 | | | |
| 23 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.97 | 0.96 | 0.98 | 0.98 | 0.97 | 0.99 | 1.00 | | |
| 24 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.99 | 0.98 | 0.99 | 0.97 | 0.98 | 0.98 | 0.99 | 1.00 | |
| 25 | 0.71 | 0.78 | 0.78 | 0.78 | 0.78 | 0.73 | 0.78 | 0.78 | 0.81 | 0.81 | 0.78 | 0.78 | 0.78 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.96 | 0.97 | 0.97 | 0.98 | 0.98 | 1.00 |

*Abbreviations see Table 1




1–25 see Table 1

Fig. 2. Dendrogram generated by UPGMA cluster analysis representing the genetic relationship among twenty-five accession of *Petunia* × *atkinsiana* D. Don. cv. Prism Red. The number at the branches indicate 2,000 bootstrap replications

They found that genetic distances between the 22 banana clones varied from 0.70 to 1.00. While, GADAKH et al. (2017) evaluated the genetic similarity between controls, salt, and drought-tolerant mutants of sugarcane, which ranged between 0.41 and 0.75.

## Conclusions

In conclusion, the genetic variation between non-mutaded and mutated plants of *Petunia* × *atkinsiana* D. Don cv. Prism Red was efficiently assessed with the RAPD-PCR analysis. Chemical mutagenesis, in combination with the tissue culture technique, seems quite appropriate for the improvement of ornamental plants like a petunia, and the variation could be detected at the stage of regeneration, even before hardening in the

greenhouse. This technique has proved to be very sensitive for the characterization of *in vitro*-selected, saline-tolerant, putative mutants of petunia plants.

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