Somaclonal variation in the *in vitro* regenerated pineapple (*Ananas comosus*): investigation of the cellular characteristics, biochemical specificities and ISSR markers

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Abstract. Several parameters of the *in vitro* regenerated, grown and adapted to the greenhouse plantlets were studied and compared ($P \leq 0.05$). The mean length of stem and leaves of the *in vitro* regenerated pineapple significantly decreased under greenhouse conditions. The chlorophyll pigments content in the regenerated plantlets was increased and carotenoid, anthocyanins, carbohydrate, total phenols, and bromelain showed a relatively lower amount in the *in vitro* plantlets. Cluster analysis was performed on the basis of Dice genetic distance matrix for the thirty regenerated plants, as well as the mother plant. On the basis of the Neighbor Joining Cluster the plantlets were grouped into three main clusters. AMOVA test showed that 46% of the genetic variance is within the groups, while 54% of the rest belongs in-between the three groups. On the basis of this analysis a significant genetic difference was observed within the regenerated plantlets (P=0.01).

Key word: ISSR, micropropagation, regenerated plantlet, somaclonal variation

Introduction

Pineapple belongs to the *Bromeliaceae* family. As a tropical fruit, the plant plays an important commercial role in the economy of a number of tropical countries. The most generally applied method of pineapple propagation so far has been through vegetative regeneration. Traditional propagation uses various vegetative parts, organs, or tissues, such as suckers, hapas, leafy branches, slips, crowns, and butts or stumps from the mature plant (Rangan 1984). Aghion & Beauchesne (1960) were the first to report the use of *in vitro* micropropagation method. Numerous papers have been published on this subject ever since. Plants were regenerated *in vitro* from the apex or axillary buds of the crown (Fitchet 1985), slips (Sita & al. 1974), lateral buds (Zepeda & Sagawa 1981), syncarp (Wakasa 1979), leaf bud (Seow & Wee 1970), as well as the callus (Rao & al. 1981). Some of these methods have also induced variations (DeWald 1988; Wakasa 1979). The appearance of off-types was mainly due to somaclonal variation (Larkin & Scowcroft 1981). These variants may provide useful characteristics, such as new leaf and fruit shape, leaf color, and spininess (Kiss & al. 1992). Maintenance of the genotypic and phenotypic identity is an indispensable requirement for mass propagation.

In order to obtain high-quality transplants at an increased rate by micropropagation, optimization of the culture parameters is of prime importance. Culture condition, especially sucrose as a growth regulator, influences photosynthesis in the *in vitro* growing plants. Evaluation of the photosynthetic properties is essential for optimization of the culture condition, in order to achieve an efficient micropropagation. The chlorophyll content of the plant has been used as a

mean value for evaluating the photosynthetic capacity of micropropagated plants with difficulties in the measuring system (Ibaraki 2006). It has been assumed that photosynthetic activity is related to the content of photosynthetic pigments and also to chlorophyll a and chlorophyll b content (MacIntyre & al. 2002). The chlorophyll molecules, which trap light and transfer energy required for driving the photochemical reactions, are some of the most photochemically active compounds in the photosynthesis, the determination of which in the regenerated plants would provide useful information regarding the plant's photosynthetic status during a micropropagation procedure.

In recent years, the extent of somaclonal variation in plants has been reported by application of molecular markers (Aversano & al. 2009). Lack of polymorphism associated with the *in vitro* regeneration was reported in tomato (Smulders & al. 1995), Norway Spruce (Fourré & al. 1997), oil palm (Rival & al. 1998), begonia (Bouman & De Klerk 2001), almond (Martins & al. 2004), potato (Sharma & al. 2007), banana (Sheidai & al. 2009), and cotton (Sheidai & al. 2012) by application of random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) markers, respectively. In contrast, major differences were found in alfalfa (Piccioni & al. 1997), *Codonopsis lanceolata* (Guo & al. 2006), wild pear (Palombi & al. 2007), and in cotton (Sheidai & al. 2014) using RAPD and ISSR markers.

Feng & al. (2013) have developed microsatellite markers for pineapple. Also, Zhou and co-workers (2015) have developed 213 single nucleotide polymorphism (SNP) markers. They have shown a high rate of duplicates in the studied pineapple collection. Different molecular markers such as AFLP, SSR, EST-SSR, SNP, RFLP, and ISSR were used for evaluation of the genetic variation among *Ananas comosus* L. cultivars (Paz & al. 2012; Zhou & al. 2015; Duval & al. 2001; Vanijajiva 2012; Feng & al. 2013). Regarding *in vitro* propagation, Perez & al. (2012) have obtained two new pineapple somaclonal variants derived from an *in vitro* culture of cv. Red Spanish Pinar (P3R5 and Dwarf). Genetic variations were observed with the help of AFLP markers, along with biochemical characteristics. The pineapple somaclonal variations have been earlier characterized by Wakasa (1977, 1989), Dewald & al. (1988), Lii & al. (1989), and Feuser & al. (2003). However, all these studies have shown only a number of characteristics, since the studies were not carried out in detail.

In the present study, our objective was to study the pineapple somaclonal variation of the *in vitro* regenerated pineapple plantlets applying the concept of biochemical and molecular markers.

Material and methods

Plant and growth conditions. The pineapple plants (*Ananas comosus* (L.) Merr.) were provided by the Plant Tissue Culture Company. Pineapple plantlets were micropropagated according to the protocol proposed by Daquinta & Benegas (1997), using the crown buds as the source of explants.

For *in vitro* plantlet cultivation, the culture medium contained Murashige and Skoog (1962) salts (MS), 1.0 mg/l naphthalenacetic acid (NAA), 3.0 mg/l 6-benzylamino purine (BA), activated charcoal (0.5 g/l), 30 g/l sucrose, and 7 g/l agar (Farahani & al. 2012). Prior to sterilization, the pH of the medium was adjusted to 5.7 for all phases of the *in vitro* cultures. All *in vitro* manipulations were performed under aseptic conditions. The culture media were sterilized by autoclaving at 121 °C at 1.5 kPa pressure. Buds were placed onto the culture medium and were maintained in 25 mm culture jars at 26 ± 2 °C; with a 16 h photoperiod (40 μmol $m^{-2}s^{-1}$ photosynthetic photon flux with cool-white fluorescent tubes).

***Biochemical studies*:** Samples of the leaves were stored in liquid nitrogen prior to biochemical analysis. Each biochemical determination started from three independent pooled samples (100 mg each). Leaves were finely ground in liquid nitrogen and by using the appropriate methods, chlorophyll a, chlorophyll b, and the total chlorophyll content were measured (Porra 1991), in addition to other biochemical constituents of the plants leaves, including carotenoids (Lichtenthaler & Wellburn, 1983), anthocyanins (Lee & al. 2005), carbohydrate (Stitt & al. 1989, Dubois & al. 1956), total phenols (based on Folin-Ciocalteu reagent, Waterhouse 2002), as well as total proteins (Lowery & al. 1951).

A raw extract was obtained from the leaves and stem of the *in vitro* cultivated pineapples, using 1 g of leaves macerated in 5 ml of 0.2 M phosphate buffer at a different pH (6.7), and filtered through passing gauze

cloths twice (Ferreira & al. 2011; Lopes & al. 2012). The total protein concentration was determined according to Bradford's method (Bradford 1976; Padilha & al. 2012).

DNA extraction and ISSR analysis

For genetic analyses, 2-3 fresh leaves from each regenerated explant, as well as mother plant were used and pooled for DNA extraction. The extraction was carried out by a DNA extraction kit (Zist Danesh Yaran, Iran), according to the manufacturer protocol. The quantity and quality of the extracted DNAs were examined by UV-spectrophotometer and 0.8 % agarose gel electrophoresis, respectively.

Ten ISSR primers $(CA)_7AC$, $(CA)_7GT$, $(GA)_9A$, $(GA)_9T$, and UBC807 $[(GA)_8T]$, UBC810 $[(AG)_8T]$, UBC811 $[(GA)_8C]$, UBC823 $[(TC)_8C]$, UBC834 $[(AG)_8YT]$, and UBC849 $[(GT)_8YA]$ commercialized by University of British Columbia were used in order to study the somaclonal variation among the *in vitro* regenerated plantlets. Di-nucleotide repeats with one or two nucleotides anchored at their 3' ends of ISSR primer sequences were used. PCR reactions were conducted in 25 µl volume reaction mixture containing 10 mM Tris- HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany), plus 0.2 µM of a single primer, 20 ng genomic DNA, and 3 U of *Taq* DNA polymerase (Bioron, Germany). We used Corbett thermocycler (CFX1-96, Australia) under the following program: 5 min initial denaturation step 94 °C, 45 s at 94 °C; 45 s at 47 °C, and 60 s at 72 °C. Finally, the reaction was completed by 5 min extension at 72 °C. ISSR banding profiles were visualized in the 1.5 % agarose gel electrophoresis followed by Syber green staining.

Statistical analyses

Thirty explants were used in the study and each experiment was repeated three times. The Statistical Package for Social Sciences (SPSS) (Version 8.0) was used for performing a one-way ANOVA test ($p \leq 0.05$).

For genetic analyses, ISSR profiles for each locus were scored as binary data (1 for presence and 0 for absence of the allele). Genetic parameters including Ne (effective allele), I (Shannon index), He (expected heterozygosity), and the percentage of polymorphism for each treatment was calculated using GenAlEx ver 6.5 program. Genetic differentiation of the regenerated, as well as of the mother plant (0 Gy, 30 Gy, and 60 Gy of radiation) was evaluated by analysis of the molecular variance (AMOVA) test using GenALEx ver. 6.5 program, and principle of coordination analysis (PCoA) (Podani 2000; Weising & al. 2005).

Results and discussion

***Morphology and cellular assay*:** The morphology, cellular, and biochemical parameters of the *in vitro* regenerated plantlets were assessed. The regenerated plantlets also exhibited a number of phenotypic differences, as compared to the mother plants (Table 1). We used 16 indicators based on a wide range of morphological parameters, including length of stem, length of branch, number of branch/plantlet, length of leaf/plantlet, and

Table 1. Phenotype effects in the regenerated plantlets and mother plants .

Mean morphological characteristics	Mother plant (8 months)	*In vitro* regenerated plantlets (8 months)	Overall percentage ratio of variation recorded in each phenotype
Length of stem (cm)	57.00 ±2.00	7.00 ±.00	12.28
Length of branch (cm)	17.00 ±.00	6.05 ±.00	35.58
Number of branch/ plantlet	2.00 ±.00	0.001 ±.00	0.05
Length of leaf/ plantlet (cm)	38.00 ±.00	6 ±.00	15.78
Color of leaf	Blue- green	Yellow- green	
Mean cellular characteristics			
Number of stomata/mm^2	10.00 ±2.00	7.00 ±.00	70
Size of stomata (×40) (µm)	68.00 ±.00	55.00 ±2.00	80.88
Size of parenchyma (×40) (µm)	98.08 ±.00	65.00 ±1.00	66.27
Mean Biochemical characteristics			
Chlorophyll a (µg/g)	0.850 ±.02	8.33 ±1	980
Chlorophyll b (µg/g)	1.5 ±.2	19 ±2	1266
Total chlorophyll (µg/g)	10 ±1	50 ±2	500
Carotenoid (µg/g)	200 ±3	0.001 ±.2	0.0005
Anthocyanin (µg/g)	20 ±1	0.002 ±.017	0.01
Carbohydrate (mg/g)	10.00 ±00	7.00 ±00	70
Phenolics content (mg/g)	1.02 ±.009	0.002 ±.013	0.19
Bromelain (mg/g)	13.00 ±.061	8.00 ±00	61.53

M: Mother plant; **R**: Regenerated plantlet
Results with the same letter are not statistically different (P≤0.05).

color of the leaves; and cellular parameters such as number of stomata/mm^2, size of stomata and size of parenchyma, in addition to biochemical characters comprising chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, anthocyanin, carbohydrate, phenolic content, and bromolin traits. These data clearly reflect the various aspects of the somaclonal variation possibly occurring in the pineapple. Furthermore, these Fig.s (Table 1) reflect the impressive effect of a small genetic modification of the phenotype caused by the *in vitro* culture (Perez & al. 2011).

Comparing the regenerated plantlets with the parental plants from which they were grown, we found significant morphological variations ($p \leq 0.05$). These morphological variations included the length of stem, leaf, and branch, number of branches, number of stomata, diameter of stomata, mean size of parenchyma cells, levels of chlorophyll a, chlorophyll b, total chlorophyll pigments, carotenoid, anthocyanin, carbohydrate, phenolics, and bromelain (Table 1). The percentage of variation for other phenotype indicators was classified into three categories: less than 20 %, between 20 and 50 %, and over 50 %. The overall percentage of variations in Table 1 indicates that the length of branch between the two plant materials significantly differed from the other parameters (above 35 %). The 8-month mother plants were taller, with leaves of less intense green and thorns on the margins (Fig. 1A). The number of leaves was almost equal in both plant types (Fig. 1B).

The length of stem, number leaves and number of branches of the *in vitro* regenerated plantlets were lower, as compared to the mother plants (Fig. 1C). Changes in the plant height and leaf size among the micropropagated plantlets have been reported by Ravindra & al. (2004) as resulting from somaclonal variation in some plant species. The high variability observed in the micropropagated rhubarb PC49 might have been triggered out by the cytokinin during micropropagation (Yipeng & al. 2005). Radhakrishnan & Kumari (2008) have reported somaclonal variation occurrence in soybean caused by hormonal concentration in the tissue culture medium.

Under greenhouse conditions, mother plants (i.e., 8-month plants) showed stem length of 57 cm, leaf length of 38 cm, and a number of two branches, while in the regenerated plantlets of the same age as mother plants, the observed mean stem length was 7 cm and the leaf length 6 cm (Fig. 2A), respectively.

Compared to mother plants, the regenerated plantlets showed significant decrease in a number of aspects, with the main difference in the number of stomata per mm^2 and comprising only 70 % of the respective number in the mother plants (i.e. 7/10). Furthermore, with respect to the size of stomata, the regenerated plantlets were found to have leaves with the mean stomata size of merely 55.65 µm. The re-

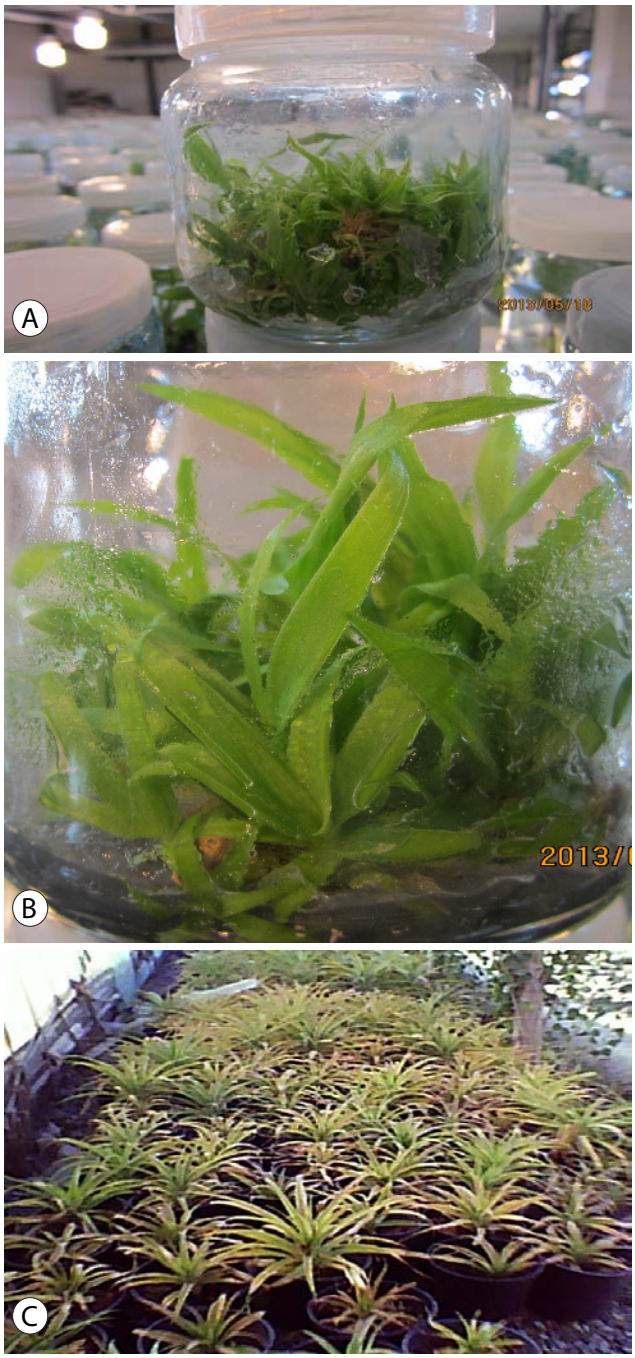

Fig. 1. A – Multiple shoots formation from the subculture bud explants. **B** – *In vitro* plantlets following to the 4th subculture; **C** – Regenerated plantlets after acclimatization in the greenhouse.

generated plantlets showed 66.3 % of the photosynthetic parenchyma size, as compared to mother plants (65.98 μm) (Fig. 2B). Architecture of the regenerated plantlets has also changed from almost wide in the mother plant to a narrower and more compact form in the regenerated plantlets. The observed results were in agreement with the report of Pérez & al. (2009).

In vitro propagation of the pineapple has also demonstrated that light is the main factor that influences the plant quality (Gonzalez & al. 2005) and ensures a better agronomic and anatomical change under greenhouse conditions (Damasceno & al. 2008).

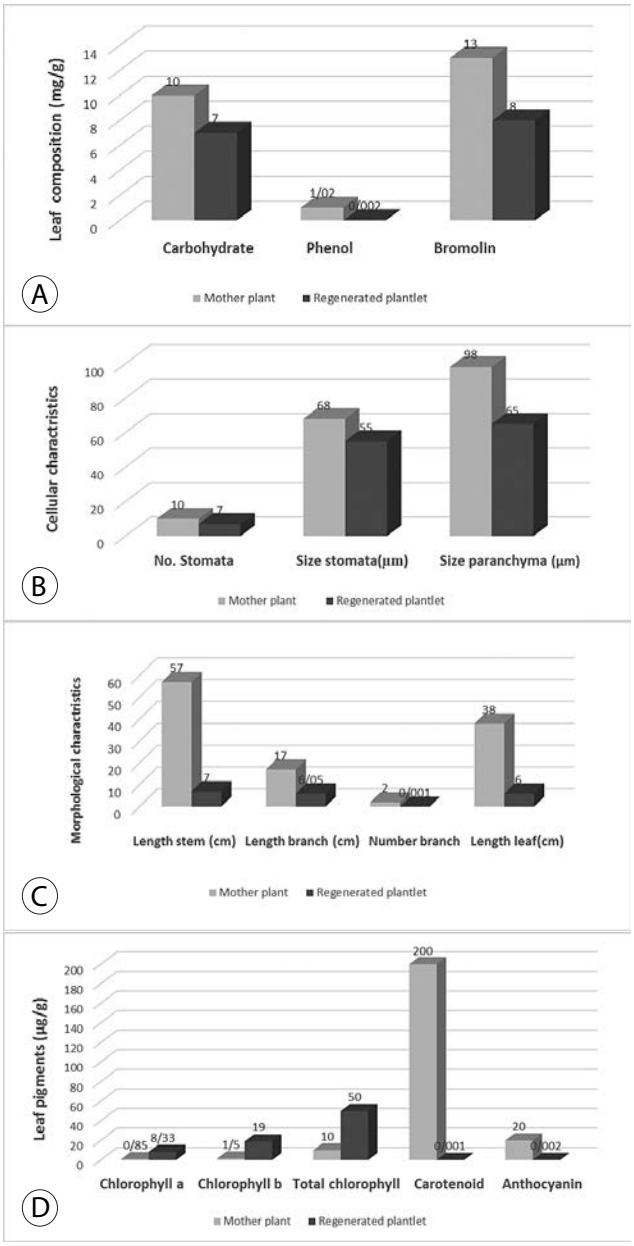

Fig. 2. Comparison between mother plants and regenerated plantlets with respect to **A**) morphological characteristics; **B**) cellular characteristics; **C**) leaf pigments; **D**) Leaf composition.

***Pigments content*:** The most dramatic and consistent differences were observed in the levels of pigments content between mother plants and regenerated plantlets (Table 1). Regenerated plantlets have markedly increased their content of pigments during subcultures. On the other hand, the content of chlorophyll a, chlorophyll b and the total chlorophyll pigments were higher in the regenerated plantlets (8.33, 19, and 50 μg/g, respectively). In contrast, carotenoid and anthocyanin contents were higher in mother plants (200 and 20 μg/g) (Fig. 2C). Such variation in chlorophyll content of the leaves was also observed in other cultures (i.e. banana and pineapple) with different intensity (Zaffari & al. 1998; Soneji & al. 2002).

Miler & al. (2014) have observed a change in color as a result of the emerging carotenoids in inflorescence of the regenerated chrysanthemum, which was not present in the control plants. In chrysanthemum cultivars regenerated from internodes, the plants have shown only true-to-type phenotypes similar to those of the control plants (Miler & al. 2014).

Generally, it is well known that changes in the environmental conditions from *in vitro* to *ex vitro* mode cause stress in the plants resulting in the accumulation of abscisic acid (ABA), phenol, proline, and reactive oxygen species (Batrova; Posposilova & Synkova 2008). However, the results obtained for *in vitro* pineapple have shown that a change in environmental conditions may possibly affect carbon metabolism (Nievola & al. 2005). Furthermore, temperature and plant height have also shown a notable influence on morphological variables in the plants cultivated in greenhouses (Damasceno & al. 2008).

***The content of carbohydrate*:** In Table 1 we have summarized the results obtained for carbohydrate content in *in vitro* regenerated plantlets and mother plants. A high level of carbohydrate content was observed in mother plants (10 mg/l). In contrast, in *in vitro* regenerated pineapple plantlets the average carbohydrate content in the mother plant (10 mg/l) was higher than in the *in vitro* plantlets (7 mg/l) (Fig. 2D). Micropropagation has caused a significant decrease in the carbohydrate content in shoots. The maximum carbohydrate content was recorded in shoots growing under greenhouse conditions.

***Phenolic content*:** The results of the total phenolic content in *in vitro* regenerated plantlets and mother plants were studied as indicated in Table 1. Manipulation of the secondary product formation in the medicinal plants has been made possible by varying the culture conditions, including the growth regulator type and concentration (Zhi & al. 2005; Ferreira & al. 2011; Apte & al. 1979). The type and concentration of auxin or cytokinin, as well as the modified ratio of auxin/cytokinin affects the concentration of secondary metabolites in the plant cells and tissue cultures (Palacio & al. 2012).

Analysis of the total phenolic content in *in vitro* regenerated plantlets and in mother plants has indicated a significant amount of phenolic compounds in the mother plants. The total phenolic content is relatively low in the *in vitro* pineapple plantlets (0.002 mg/g) in comparison to the mother plants with the highest amount of phenolic content of 1.02 mg/g (Fig. 2D). Palacio & al. (2012) have worked on micropropagation of *Larrea divaricata* (Cav.) and have shown a lower production of phenols in the tissue culture systems, as compared to the mother plants.

A different concentration of naphtalenacetic acid and benzylaminopurine has significantly affected exudation of the phenolic compounds from explants into the culture medium. The media with the highest PGR concentration (NAA and BAP) were found to contain the highest phenol content (North & al. 2012).

According to Lux-Endrich & al. (2000), phenols are reactive compounds synthesized in plant tissues. Furthermore, Chamandoosti (2010) has reported a relationship between the chemical composition of the media and phenolic leakage, media discoloration, and explant browning and death. In other related studies, nathalenacetic acid and benzylaminopurine were reported to play an important role in the biosynthesis of secondary metabolites in the *in vitro* culture (Shilpashree & Rai, 2009). Therefore, the total content of phenolic compounds in tissue culture can be minimized with selection of suitable medium constituents.

Activated charcoal significantly reduces phenolic content in the culture medium. Similar to our results, Birmeta & Welander (2004) have reported that activated charcoal is more effective in reducing polyphenol exudation in the *Ensete ventricosum* (Musaceae). Several researchers have also reported the usefulness of activated charcoal in controlling the oxidative browning which is associated with phenol production of the explants in tissue culture (North & al. 2010; North & al. 2011). Incorporation of activated charcoal into the medium is an established method as the most effective way for controlling polyphenol exudation (Diro & van Staden 2004; Kiong & al. 2007). The adsorption of phenols in the medium prevents the browning of tissues (Madhusudhanan & Rahiman 2000; Chawla 2002).

***Bromelain content*:** Table 1 presents the results of measurement of bromelain content. In mother plants, 13 mg/l of bromelain was recorded, while in *in vitro* regenerated pineapple plantlets the corresponding content was 8 mg/l, and lower, when a buffer solution with basic pH was used for measurement (Fig. 2D). Alcantara & al. (2011) and Pereira & al. (2011) have found that extended time affects negatively the *in vitro* explant aspects, leading to a decrease in plant survival during acclimatization in *Eucalyptus grandis, Eucalyptus urophylla* and in apples. The earlier results obtained on *in vitro* pineapple have shown that it is possible to alter carbon metabolism by changing the environmental conditions during the growth period (Nievola & al. 2005). Apte & al. (1979) have also correlated the significant differences observed in the bromelain content during various stages of growth (i.e. culture time), with the increased activity at the 8^{th} month indicating that growth characteristics influence strongly the formation of enzymes. Thus, the differentiation process may play a role in the formation of proteolytic enzymes. According to Piza & al. (2002), the stages of plant development during growth also exercise an influence on protein synthesis. Thus, results presented in this report are in accordance with those previously obtained (Piza & al. 2002), adding that the proteolytic activity of the enzyme (i.e. bromelain) depends not only on the plant but also on the anatomical site in the plant (i.e. the type of plant tissue) that was used for analysis. These results suggest that culture conditions can induce genetic overexpression or enhance the efficiency of the enzyme's catalytic activity involved in the lysis of primary metabolites in plants. Vilanova Neta & al. (2012) have reported that *in vitro* regenerated plantlets (Pérola cultivar) have higher total protein content than the mature plants (Apte & al. 1979). Thus, it may turn promising for shoots and plants regeneration in the subsequent phases, with efficient micropropagation followed by transfer of crops to the field.

The applied environmental conditions have caused an obvious phenotypic divergence in the studied pineapple plants. The effects of increased light intensity and gradual reduction of the relative humidity at an early stage were demonstrated in pineapple plantlets (*Ananas comosus*) by Silva & al. (2008). Batagin & al. (2009) have found an increased cuticle thickness, wavy contours of the epidermal cells. Distribution, and quantity of mesophyll fibers during the acclimatization process evidenced the interfering effect of light on the morphological characteristics of pineapple plantlets. *In vitro* propagation of the pineapple has demonstrated that light is the factor which highly influences the plant quality (Gonzalez & al. 2005) and provides for a better agronomic, and anatomical change during the acclimatization phase (Damasceno; Souza & Gomes 2008). Chen & al. (2006) have studied several *Syngonium podophylum* somaclonal variants, within which small genetic differences along with remarkable phenotypic modifications were also observed. Similar results were recorded by Prado & al. (2007) in *Actinidia deliciosa* somaclonal variants.

Somaclonal variation has been associated with changes in chromosome number and structure, point mutations, DNA methylation (Brown & al. 1993), transposon activation, deletion, genome rearrangement, polyploidy, or nucleotide substitution (Bhatia & al. 2005). However, not much has been published about the effects of somaclonal variation at morphological and physiological levels. At this point of our investigations, it is difficult to say which genes are involved in the observed morphological and physiological changes.

Table 2. Genetic parameters assessment based on ISSR data studied in the regenerated pineapple plantlets.

ISSR locus	Ne	I	He
UBC810	1.447	0.480	0.304
UBC807	1.301	0.388	0.229
UBC811	1.366	0.428	0.262
UBC823	1.213	0.306	0.170
UBC849	1.207	0.310	0.170
UBC834	1.329	0.406	0.244
CA7AC	1.220	0.319	0.177
GA9A	1.237	0.331	0.187
GA9T	1.367	0.416	0.256
CA7GT	1.401	0.453	0.282
Mean	1.318	0.391	0.234
SE	0.007	0.005	0.004

Table 3. AMOVA test based on ISSR data. Groups are according to NJ cluster analysis (Fig.3).

Source	df	SS	MS	Est. var.	%
Among Pops	1	255.558	255.558	52.104	46%
Within Pops	30	1805.067	60.169	60.169	54%
Total	31	2060.625		112.273	100%
Stat	**Value**	**P(rand >= data)**			
PhiPT	0.464	0.010			

df= degree of freedom, **SS**= sum of square, **MS**= mean of square.

***Molecular analysis*:** Ten ISSR loci that produced reproducible alleles were considered for further analyses. All loci showed 100 percent polymorphic bands with 343 total bands. The highest genetic parameters including the number of effective alleles (Ne), Shannon index (I) and Nei s' genetic diversity (He) were observed in UBC810 locus, while UBC849 and UBC823 loci showed the lowest genetic parameters (Table 2).

Cluster analysis was performed on the basis of Dice genetic distance matrix for thirty regenerated pineapple plantlets, as well as for the mother plant (Fig. 3). On the basis of Neighbor Joining Cluster, plantlets were grouped into three main clusters. Ten of the 30 regenerated plantlets were grouped in the first cluster, while nine regenerated plantlets were distinctively grouped in the second cluster. Meanwhile, plantlets in the third cluster were divided into two sub-clusters. The mother plant (No 31) was grouped with the regenerated plantlets Nos 1, 6, 8, 11, and 17 and was shown to be more similar to the *in vitro* plantlets. However, molecular analysis has profoundly shown genetic variation among the regenerated plantlets and has supported the existence of somaclonal variation in *in vitro* regenerated plantlets.

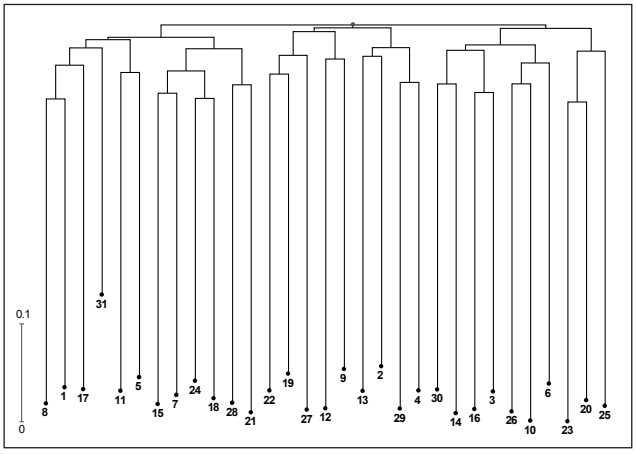

Fig. 3. Neighbor Joining Cluster based on ISSR markers. Numbers (Nos) 1 to 30 are the regenerated pineapple plantlets and number 31 is the mother plant.

Genetic differences between the three clusters as provided by NJ clustering were identified by the analysis of molecular variance (AMOVA) of ISSR data (Table 3). AMOVA test showed that 46 % of the genetic variance was contributed within the groups, while 54 % of the variance took place between the three groups. On the basis of this analysis a significant genetic differentiation was recorded between the regenerated pineapple plantlets (P=0.01).

Similarly to the obtained morphological, cellular and biochemical results, our molecular findings have also revealed variation among the *in vitro* regenerated plants, which provides further evidence on the existence of somaclonal variation in pineapple plantlets. On the other hand, the ISSR loci have shown genetic difference between the mother plant and the regenerated pineapple plantlets as further support for the other findings in the present study.

Recently, ISSR markers have been broadly used for identifying somaclonal variation in different plants, such as yacon (*Smallanthus sonchifolius*), pepper, apple, and rice (Viehmannova & al. 2014, Bello-Bello & al. 2014, Ngezahayo & al. 2007, Noormohammadi & al. 2015).

In a study of somaclonal variation of the pineapple cultivars, Feuser & al. (2003) have detected low genetic variation among the micropropagated pineapple plantlets by using isozyme (average 0.67 %) and RAPDs (average 7.5 %). They reported genetic fidelity of the micropropagated pineapple plantlets. On the other hand, Perez & al. (2012) have studied Red Spanish Pinar (as a donor) P3R5 and Dwarf Pineapple variants and reported genetic distance between the somaclonal variants and the donor, but not too significant. However, different types of genetic variation were observed between them. Although low levels of genetic variation were reported in this study, morphological, physiological, and biochemical characterization showed some significant differences between the donor plants and variants (Perez & al. 2012).

Our findings have shown profound genetic variation among the regenerated pineapple plantlets. The ISSR polymorphism observed in the regenerated pineapple plantlets tissue culture indicates genetic polymorphism in genotypes as well. Genetic, morphological, biochemical, and physiological variations among the regenerated plants of the single subculture indicate the occurrence of somaclonal variation.

Studies carried out by Feuser & al. (2003) and Perez & al. (2012) have revealed genetic fidelity among pineapple plantlets, while our findings show high genetic diversity among the regenerated and mother plants. These reports supported the effect of main factors in somaclonal variation, including genotypes, molecular markers, and culture media. In the present study, we have reported somaclonal variation regarding different aspects, such as genetic, biochemical and morphological characteristics. Such variations could be taken into consideration in the breeding of this commercially important plant.

Conclusion

By *in vitro* propagation of the pineapple we demonstrated that light has the greatest influence on the plant quality and provides for a better agronomic and anatomical change under greenhouse conditions. Our molecular findings, similarly to the obtained morphological, cellular and biochemical observations, have revealed variations among the *in vitro* regenerated plantlets, which provide further evidence on the existence of somaclonal variation in the pineapple plants. On the other hand, ISSR loci analysis showed genetic differences between the mother plant and the regenerated plantlets, a finding that further supports other results obtained in the present study.

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