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New Pineapple Somaclonal Variants: P3R5 and Dwarf

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ABSTRACT

The Food and Agriculture Organization has highlighted pineapple as one of the most important tropical fruits. Since classical pineapple breeding is difficult, biotechnology has emerged as an attractive instrument. We obtained two new pineapple somaclonal variants derived from *in vitro* culture of cv. Red Spanish Pinar: P3R5 and Dwarf. The AFLP analysis revealed an existing genetic distance. So far 44 phenotype indicators selected due to their relation to a wide range of important agricultural, morphological and physiological processes have been evaluated. P3R5 differed from the donor in 19 variables (19/44; 43.18%), while Dwarf varied in 31 indicators (31/44; 70.45%). The number of shoots was significantly different among the three plant materials. Dwarf showed two shoots per plant while P3R5 and the donor did not form any shoots. We also observed that water use efficiency, chlorophyll b concentration, total chlorophyll concentration, transpiration rate, chlorophyll a concentration, thickness of leaf photosynthetic parenchyma, fruit mass with crown, content of free phenolics and superoxide dismutase specific activity were also very different among the three plant materials. The Euclidean distances of each somaclonal variant to the donor plant material taking into consideration the genotype (AFLP) and the phenotype evaluations were also calculated. Regarding the genotype information, P3R5 is separated from cv. Red Spanish Pinar by 2.83 units of Euclidean distance, and Dwarf by 3.00 units. However, the phenotype indicators revealed higher differences: 3.74 in P3R5 and 4.71 in Dwarf. To our knowledge, this is the first report of a comprehensive analysis of pineapple somaclonal variants.

Keywords: *Ananas comosus* (L.) Merr.; Genotype Variation; Phenotype Variation

1. Introduction

Pineapple belongs to the *Bromeliaceae* family and is one of the most economically important tropical fruit. The interest for its production is due to the high cost that reaches in the fresh fruit and industrialized markets, its great food value, its pleasant taste and its beauty for commercialization. The worldwide production in 2008 was 19.16 million tons [1]. Because of this, several research groups are developing basic and applied studies to create new varieties with better agronomic performance. Pineapple breeding using naturally occurring genetic variation and conventional methods has succeeded in several countries.

In 1914, the Pineapple Growers Association of Hawaii started one of earliest and most concerted efforts in pineapple improvement. One of the main objectives was to develop pest and disease resistance in Smooth Cayenne [2]. Many other countries have also started hybridization programmes to develop high-yielding varieties with specific adaptation to their own environments, for instance, in Taiwan [3,4], Malaysia [5-7], The Philippines, Cote d'Ivoire, Puerto Rico, Cuba and Australia [8-15]. The varieties obtained recently by hybridization programs in Brazil (Ajubá, Imperial), Australia (Aus-Carnival and Aus-Jubilee), USA (Honey Gold and MD2), Martinique (FLHORAN41) and Malaysia (Josapine) [16].

As classical pineapple breeding is extremely laborious and time-consuming [17], biotechnology is an attractive tool for improving elite clones [18-23]. In this context, some results have been obtained with somaclonal variation.

Genetic variation is very important in crop improvement and forms the basis of development of new varieties. Somaclonal variation is a valuable tool in plant breeding wherein variation in tissue culture regenerated plants from somatic cells can be used in the development of crops with novel traits [24]. Larkin and Scowcroft [25] were the first researchers to demonstrate and coin the term somaclonal variation. Variations may pre-exist in the natural population of plants from field collection or genebank or it may develop as a result of tissue culture conditions [26].

In recent years a number of studies have measured, through molecular markers, the extent of somaclonal

variation in plants [27]. Lack of polymorphisms associated with *in vitro* regeneration was reported in tomato [28], Norway spruce [29], oil palm [30], begonia [31], almond [32], and potato [33,34] using RAPD, ISSR and AFLP markers. By contrast, major differences were found in alfalfa [35], in *Codonopsis lanceolata* [36] and wild pear [37] using RAPD and ISSR markers.

Pineapple somaclonal variations have been previously characterized [38-42]. However, all these studies showed only few characters and were not studied in detail as only the number of leaves per plant; the number of thorns per leaf and leaf color were reported.

The present study culturing *in vitro* pineapple axillary buds with naphthalene acetic acid and 6-benziladenine for micropropagation; kinetin to induce callus formation; and indole-3-butyric acid and gibberellic acid for plant regeneration, was carried out to check possible genetic alterations in the plants produced. These growth regulators were the only putative mutagenic agents we used.

We obtained two new pineapple somaclonal variants derived from *in vitro* culture of cv. Red Spanish Pinar [43,44]. This paper shows a broad genotypic and phenotypic analysis of P3R5 and Dwarf somaclonal variants.

2. Materials and Methods

2.1. Plant Material, Media and Culture Conditions

Fifty pineapple buds (cv. Red Spanish Pinar, donor) were collected from field-grown plants and cultured following the protocol described by Daquinta and Benegas [45]. Explants were placed in conventional plant containers for micropropagation (300 ml) where 25 ml liquid culture medium fed five explants. The culture medium included Murashige and Skoog salts [46], 100 $mg \cdot l^{-1}$ myo-inositol, 0.1 $mg \cdot l^{-1}$ thiamine-HCl, 30 $g \cdot l^{-1}$ sucrose, 4.4 μM 6-benzyladenine and 5.3 μM naphthalene-acetic acid. Forty-three young pineapple shoots were obtained after 42 d of bud implantation.

2.2. Multiplication Culture Medium

Shoots were transferred to the multiplication culture medium (as described above except: 9.3 μM 6-benziladenine and 1.6 μM naphthalene-acetic acid). They were subcultured at 42 d intervals for 168 d. Twenty-four thousand seven hundred and sixty-eight shoots were then obtained.

2.3. Callus Induction

Three hundred young leaves were randomly selected as explants for callus formation. The culture medium included: Murashige and Skoog salts [46], 100 $mg \cdot l^{-1}$ myoinositol, 0.1 $mg \cdot l^{-1}$ thiamine-HCl, 30 $g \cdot l^{-1}$ sucrose, 29.0 μM naphthalene-acetic acid and 9.7 μM kinetin. The calli were proliferated for 4 months with subcultures every 30 d.

2.4. Plant Regeneration

Five hundred calli (Ø: 3 mm) were randomly selected and transferred to the plantlet regeneration medium: Murashige and Skoog salts [46], 100 $mg \cdot l^{-1}$ myo-inositol, 0.1 $mg \cdot l^{-1}$ thiamine-HCl, 30 $g \cdot l^{-1}$ sucrose, 0.9 μM indole-3-butyric acid, 1.1 μM 6-benzyladenine and 0.09 μM gibberellic acid. Four hundred and twenty-seven *in vitro*-plantlets were obtained and later hardened in *ex vitro* for 6 months [47].

2.5. Hardening and Field Conditions

For *ex vitro* hardening, plantlets were placed in plastic trays containing 82 cm^3 of a mixture zeolite + filter cake (1:1). Microject automated irrigations for 25 s every 30 min were applied. Plantlets were kept under a photosynthetic photon flux density of 458 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Standard phytosanitary controls were applied. After hardening of *in vitro*-plantlets, 387 plantlets were transferred to the field environment and asexually propagated for two generations (30 months). The donor cultivar was used as a control. Two phenotype variants were then identified: P3R5 and Dwarf. A more detailed study was carried out to compare these two variants with the donor plant (cv. Red Spanish Pinar, plant material never cultured *in vitro*). The experiment was developed in the Field Experimental Station at the Bioplant Centre. A random block design was implemented (80 plants/clone). Field management was performed according to instructions recommended by the Cuban Ministry for Agriculture. The most important pineapple phenotypic traits were recorded during 18 months. The agricultural evaluations were made in field conditions.

2.6. Plant Material and DNA Extraction

In the second generation, in order to perform the AFLP characterization, young leaves of the donor genotype (cv. Red Spanish Pinar) and the two variants (P3R5 and Dwarf) were collected. Samples were stored at –20˚C until DNA extraction. Extraction started from 250 mg fresh mass that were finely grounded in liquid nitrogen. Extraction buffer (650 μl) was then added. It included: Tris-Cl (pH 7.5, 50 $mmol \cdot l^{-1}$), ethylene-diamine-tetra-acetic acid (20 $mmol \cdot l^{-1}$), sodium chloride (0.3 $mmol \cdot l^{-1}$), sarcosil (2%), sodium dodecyl sulfate (0.5%) and urea (4.8 $mmol \cdot l^{-1}$). A mixture (650 μl) of phenol:chloroform: isoamyl alcohol (25:24:1, v:v:v) was added. Samples were centrifuged for 15 min at 12,000 rpm at room temperature. Supernatants were collected and the pellets,

discarded. Isopropanol (0.8 volumes) was added. Samples were shortly shaken in a vortex and incubated for 10 min at room temperature. Samples were centrifuged for 10 min at 12,000 rpm. Supernatant was discarded and the pellet was washed with ethanol (70%). DNA was dried (vacuum) and dissolved in 50 µl water supplemented with 10 $\mu g \cdot ml^{-1}$ RNase A. DNA integrity and purity was checked by electrophoresis in agarose gels (0.8%) stained with ethidium bromide. Concentration was estimated visually by comparison with standards (100 - 1000 $ng \cdot \mu l^{-1}$). Concentrations of DNA samples were adjusted to 500 $ng \cdot \mu l^{-1}$.

2.7. AFLP Analysis

AFLP technique was carried out [48]. The digestion of genome DNA, the pre-amplification with a selective base and the selective amplification was performed [47]. Autoradiographs were analyzed visually to build a dicotomic numerical matrix: the number one was assigned when the band was present while zero was assigned when absent. We disregarded all weak and low peak AFLP bands. The matrix was processed with the NTSYS-pc software [49]. The simple matching index was used to create a matrix of similarity. From this matrix, a matrix of genetic distance was obtained. The UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method was used to generate a dendogram.

2.8. Morphological, Physiological and Biochemical Determination

In a subsequent procedure, the three plant materials were transferred to the Pineapple Germplasm Bank at the Bioplant Centre in a random block design. Plants grew for 6 months and then *D* leaves [50] of Red Spanish Pinar (donor), P3R5 and Dwarf were collected. Ten plants per genotype were studied (one leaf per plant). The stoma diameter, number of stomata per mm^2, diameter of leaf vascular tissue, thickness of the leaf aquiferous parenchyma, and thickness of the leaf photosynthetic parenchyma were measured [51]. The photosynthetic rate, the transpiration rate, the water use efficiency, and the internal leaf CO_2 concentration were recorded using a Portable CIRAS-2 Photosynthesis System (Europe, PP Systems, UK); covering with the leaf, the whole area of the cuvette (PLC6, 2.5 cm^2). The carbon dioxide concentration and the relative humidity of the air entering the cuvette were 375 $\mu mol \cdot mol^{-1}$ and 80% respectively, under environmental temperature (25˚C - 27˚C). Prior to obtaining the experimental data, we measured the maximum light intensity at which photosynthesis was stable which was attained at 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

To determine the levels of chlorophyll pigments (a, b, total), leaves were thinly grounded in liquid nitrogen. Evaluations were made [52]. Extraction was carried out with 5.0 ml acetone (80%, v:v). The samples were centrifuged (12,000 rpm, 4˚C, 15 min), supernatants collected and absorbances at 647 and 664 nm were recorded.

Contents of malondialdehyde and other aldehydes [53]; and phenolics (cell wall-linked, free, and total were determined [54]. Total protein contents were recorded [55]. Enzymatic activities and specific activities of phenylalanine ammonia-lyase [56], superoxide dismutase [57], and phosphoenol pyruvate carboxylase [58,59] were also measured.

2.9. Data Analysis

The Statistical Package for Social Sciences (Version 8.0 for Windows, SPSS Inc.) was used to perform One-Way ANOVA and Tukey tests ($P \leq 0.05$). The Euclidean distances of each somaclonal variant to the donor plant material were calculated. Data were standardized to vary from 0 to 1 [60].

3. Results and Discussion

3.1. Agricultural and AFLP Characterization

Only two plant materials were found to be "solid" somaclonal variants after studying three vegetative generations (P3R5 and Dwarf) and the AFLP analysis, which represents 0.52% (2 somaclonal variants/387 plants transferred to the field). The dendogram generated with the AFLP information revealed an existing genetic distance among the somaclonal variants and the donor plant [43]. The genetic distances among the three plant materials are not too significant. However, as they have different banding patterns, they are different at the genetic level.

The agricultural characterization of the third vegetative generation in **Tables 1(a)-(b)** and **Figure 1** shows that, in comparison with the donor plant (cv. Red Spanish Pinar), the variant P3R5 showed differences in the number of slips and suckers, and in the presence of thorns in the leaves and in the fruit crowns. The somaclonal variant Dwarf was different from the donor plant in regard to the plant height; the peduncle diameter; the number of shoots, slips and suckers; the fruit mass with crown; the number of eyes in the fruit; the fruit height and diameter; the leaf color; the plant architecture; the length of plant generation cycle; and the fruit color and shape (**Table 1(a)**, agricultural characterization of the third vegetative generation).

3.2. Morphological, Physiological and Biochemical Characterization

The morphological, physiological and biochemical characterization of *D* leaves in **Table 1(b)** shows that, in comparison with the donor plant (cv. Red Spanish

Table 1. Phenotypic characterization of pineapple somaclonal variants. (a) Agricultural characterization of the third vegetative generation; (b) Morphological, physiological and biochemical characterization of *D* leaves.

(a)

Phenotype indicator	Plant materials			Overall coefficients of variation recorded in each phenotype indicator (%)**
	Cv. Red Spanish Pinar (*Donor*)	*P3R5*	*Dwarf*	
Plant height (cm)*	72.0 a	73.3 a	33.2 b	38.30
Peduncle diameter (cm)*	2.6 a	2.7 a	1.6 b	26.45
Number of shoots*	0.0 b	0.0 b	2.0 a	173.21
Number of slips*	4.0 b	7.0 a	6.0 a	26.96
Number of suckers*	1.0 b	2.0 a	2.0 a	34.64
Fruit mass with crown (kg)*	1.6 a	1.7 a	0.6 b	46.79
Number of eyes in the fruit*	81.0 a	81.0 a	50.0 b	25.33
Fruit height (cm)*	15.2 a	15.6 a	9.3 b	26.39
Fruit diameter (cm)*	16.3 a	16.3 a	9.3 b	28.94
Number of crowns in the fruit*	1.0 a	1.0 a	1.0 a	0.00
Fruit content of vitamin C (%)*	18.0 a	18.6 a	17.8 a	2.30
Fruit acidity (%)*	0.3 a	0.3 a	0.3 a	0.00
Plant generation cycle (months)*	17.0 a	17.0 a	16.0 b	3.46
Presence of thorns in leaves	Many (all over the leaf edge)	Few (only on leaf extreme)	Many (all over the leaf edge)	---
Leaf color	Greenish with red zones	Greenish with red zones	Greenish	---
Plant architecture	Lightly wide	Lightly wide	Compact	---
Shape of fruit eyes	Rectangular	Rectangular	Rectangular	---
Fruit color	Red-orange	Red-orange	Yellow-green	---
Fruit shape	Tonel	Tonel	Cylindrical-Block	---
Presence of thorns in fruit crowns	Many (all over the leaflet edge)	Few (only on leaflet extreme)	Many (all over the leaflet edge)	---

**Overall coefficient of variation = (Standard deviation/Average) × 100. To calculate this coefficient, average values of the donor plant material, P3R5 and Dwarf were considered. The higher differences among the three plant materials compared, the higher the overall coefficient of variation.

(b)

Phenotype indicator	Plant materials			Overall coefficients of variation recorded in each phenotype indicator (%)**
	Cv. Red Spanish Pinar (*Donor*)	*P3R5*	*Dwarf*	
Stoma diameter (μm)*	28.2 ab	24.0 b	30.1 a	11.38
Number of stomata per mm^{2*}	110.1 a	99.3 b	84.0 c	13.41
Diameter of leaf vascular tissue (μm)*	38.1 a	32.1 b	17.2 c	36.94
Thickness of the leaf aquiferous parenchyma (μm)*	119.1 a	86.4 b	43.8 c	45.44
Thickness of the leaf photosynthetic parenchyma (μm)*	57.6 a	33.6 b	19.9 c	51.53
Photosynthetic rate (μmol CO_2 $m^{-2}\cdot s^{-1}$)*	20.2 b	19.0 b	21.7 a	6.66
Transpiration rate (mmol H_2O $m^{-2}\cdot s^{-1}$)*	41.6 a	11.5 b	45.7 a	56.70
Water use efficiency (mmol CO_2 $mol^{-1}\cdot H_2O$)*	0.7 b	1.8 a	0.5 c	70.00

Continued

Internal leaf CO_2 concentration (μmol CO_2 mol^{-1})*	222.8 c	373.7 a	253.4 b	28.16
Total chlorophyll concentration ($mg \cdot g^{-1}$ fresh weight)*	17.8 b	35.5 a	11.7 c	57.06
Chlorophyll a concentration ($\mu g \cdot g^{-1}$ fresh weight)*	10.7 b	21.3 a	7.7 c	53.99
Chlorophyll b concentration ($\mu g \cdot g^{-1}$ fresh weight)*	6.5 b	14.2 a	4.0c	64.57
Malondialdehyde content ($\mu mol \cdot g^{-1}$ fresh leaf mass)*	8.85 b	10.71 ab	12.84 a	18.45
Other aldehyde content ($\mu mol \cdot g^{-1}$ fresh leaf mass)*	120.86 a	76.06 b	109.82 a	22.83
Content of cell wall-linked phenolics ($mg \cdot g^{-1}$ fresh leaf mass)*	6370.42 ab	7594.09 a	5333.39 b	17.59
Content of free phenolics ($mg \cdot g^{-1}$ fresh leaf mass)*	1273.44 a	472.58 b	857.83 ab	46.15
Total content of phenolics ($mg \cdot g^{-1}$ fresh leaf mass)*	7643.86 a	8066.66 a	6191.22 b	13.47
Protein content ($\mu g \cdot mg^{-1}$ fresh leaf mass)*	0.0092 b	0.0124 a	0.0122 a	15.85
Phenylalanine ammonia-lyase activity ($U \cdot g^{-1}$ fresh leaf mass)*	0.29 c	0.42 b	0.62 a	37.16
Phenylalanine ammonia-lyase specific activity ($U \cdot mg^{-1}$ of protein)*	0.0071 c	0.0109 b	0.0157 a	38.15
Superoxide dismutase activity ($U \cdot mg^{-1}$ fresh leaf mass)*	0.85 a	0.90 a	0.33 b	45.36
Superoxide dismutase specific activity ($U \cdot mg^{-1}$ of protein)*	21.16 a	23.17 a	8.27 b	46.10
Phosphoenol pyruvate carboxylase activity ($U \cdot mg^{-1}$ fresh leaf mass)*	6.29 a	4.90 a	4.90 a	14.93
Phosphoenol pyruvate carboxylase specific activity ($U \cdot mg^{-1}$ of protein)*	156.88 a	126.16 a	122.01 a	14.11

*Results with the same letter are not statistically different (One Way ANOVA, Tukey HSD, $P > 0.05$).

Pinar), the variant P3R5 showed statistically significant differences in 15 indicators while Dwarf in 17 variables. Compared to the donor plant, P3R5 somaclonal variant showed significant low values in several aspects, but mainly in the transpiration rate that only reached 28% of the rate in the donor (11.5 mmol H_2O $m^{-2} \cdot s^{-1}$/41.6 mmol H_2O $m^{-2} \cdot s^{-1}$). Moreover, content of free phenolics in P3R5 merely represented 37% (472.58 $mg \cdot g^{-1}$ fresh leaf mass/1273.44 $mg \cdot g^{-1}$ fresh leaf mass). Significant increases were also recorded in P3R5 in comparison with cv. Red Spanish Pinar. For instance, the donor showed 39% of the water use efficiency evaluated in P3R5 (0.7 mmol CO_2 mol^{-1} H_2O/1.8 mmol CO_2 mol^{-1} H_2O) (**Table 1**).

Comparing Dwarf somaclonal variant with the donor, it only reached 35% of the thickness of the photosynthetic parenchyma of *D* leaf recorded in the donor (19.9 μm/57.6 μm), 37% of the thickness of the leaf aquiferous parenchyma (43.8 μm/119.1 μm), and 39% of the superoxide dismutase activity and specific activity (0.33 $U \cdot mg^{-1}$ fresh leaf mass/0.85 $U \cdot mg^{-1}$ fresh leaf mass, 8.27 $U \cdot mg^{-1}$ of protein/21.16 $U \cdot mg^{-1}$ of protein, respectively). On the other hand, the donor plant material only showed about 46% of the phenylalanine ammonia-lyase activity and specific activity (0.29 $U \cdot mg^{-1}$ fresh leaf mass/0.62 $U \cdot mg^{-1}$ fresh leaf mass, 0.0071 $U \cdot mg^{-1}$ of protein/0.0157 $U \cdot mg^{-1}$ of protein) (**Table 1**).

Changes in the above mentioned morphological, physiological and biochemical indicators have been frequently studied when plants have been submitted to different sources of stress. Reference [61], recorded the optimization of CO_2 gain through stomatal aperture while minimizing water loss in rice. The effects of flooding and drought stress on citrus seedlings physiology were measured [62]. The response of cucumber seedlings to drought stress also were measured [63]. However, to our knowledge, the effects of somaclonal variation on plant physiology have not been deeply studied. Further studies are required to elucidate the mechanisms that explain the differences observed in P3R5 and Dwarf somaclonal variants.

Figure 1. Pineapple material of Red Spanish Pinar (Donor), P3R5 and Dwarf somaclonal variants. (a) Plants at 6 months of growth under controlled environment; (b) *D* leaves of 6 month-old plants grown under the field environment; (c) Fruits just harvested in the field.

3.3. General Variation: Overall Coefficients of Variation and Euclidean Distances

The overall coefficients of variation in **Table 1** indicate that the number of shoots was significantly different among the three plant materials (173.21%).

Dwarf showed two shoots per plant while P3R5 and the donor did not form any shoot. We classified the overall coefficients of variation of the other phenotype indicators in three categories: less than 23%, between 23 and 46%, and over 46%. Then we observed that water use efficiency, chlorophyll b concentration, total chlorophyll concentration, transpiration rate, chlorophyll a concentration, thickness of the leaf photosynthetic parenchyma, fruit mass with crown, free phenolics content and superoxide dismutase specific activity were also very different among the three plant materials. However, other aldehyde content, malondialdehyde content, content of cell wall-linked phenolics, protein content, phosphoenol pyruvate carboxylase activity, phosphoenol pyruvate carboxylase specific activity, total content of phenolics, number of stomata per mm^2, stoma diameter, photosynthetic rate, plant generation cycle, fruit content of vitamin C, number of crowns in the fruit and fruit acidity showed low variability.

Table 2 summarizes the phenotypic changes of P3R5 and Dwarf somaclonal variants with respect to the donor plant material. We have used 44 indicators based on a wide range of horticultural and physiological traits. These data clearly show the various aspects where somaclonal variation can occur in pineapple. P3R5 differed from the donor in 19 variables (19/44; 43.18%), while Dwarf in 31 indicators (31/44; 70.45%; **Table 2**).

Figure 2 shows the Euclidean distances of each somaclonal variant to the donor plant material taking into consideration the genotype (AFLP) and the phenotype evaluations. Regarding the genotype information, P3R5 is separated from cv. Red Spanish Pinar by 2.83 units of Euclidean distance, and Dwarf by 3.00 units. However, the phenotype indicators revealed bigger differences: 3.74 in P3R5 and 4.71 in Dwarf. These figures support the impressive effects on phenotype of small genetic modifications caused by *in vitro* culture. Authors studied several *Syngonium podophylum* somaclonal variants within which small genetic differences and significant phenotype modifications were also observed [64]. Similar results were recorded in *Actinidia deliciosa* somaclonal variants [65].

References [66,67] summarized that 22 cultivars had been released from somaclonal variation with improved traits including yield; plant architecture; colour; pest resistance; salt, heat and freezing tolerance. However, considering that pineapple culture through *in vitro* derived plants is in practice for a long time (over 20 years in Cuba), we have only these two (P3R5 and Dwarf) variants that are stable and thus somaclonal variation in this crop should be considered a rare event.

Somaclonal variation has been associated with changes in chromosome number and structure, point mutations, DNA methylation [68], transposon activation, deletion, genome rearrangement, polyploidy, or nucleotide substitution [69]. However, not much has been published about the effects of somaclonal variation at morphological and physiological levels.

At this point of our investigation, it is difficult to say

Table 2. Summary of phenotypic modifications of P3R5 and Dwarf somaclonal variants. Classification supported by One Way ANOVA and Tukey HSD (P = 0.05) but asterisks indicate qualitative analysis (Table 1).

Somaclonal variants	Phenotype indicators			
	Not modified with respect to the donor plant material	Decreased with respect to the donor plant material	Increased with respect to the donor plant material	Other modifications with respect to the donor plant material
P3R5	25 indicators: Plant height; Peduncle diameter; Number of shoots; Fruit mass with crown; Number of eyes in the fruit; Fruit height; Fruit diameter; Number of crowns in the fruit; Fruit content of vitamin C; Fruit acidity; Plant generation cycle; Leaf color (*); Plant architecture (*); Shape of fruit eyes (*); Fruit color (*); Fruit shape (*); Stoma diameter; Photosynthetic rate; Malondialdehyde content; Content of cell wall-linked phenolics; Total content of phenolics; Superoxide dismutase activity; Superoxide dismutase specific activity; Phosphoenol pyruvate carboxylase activity; Phosphoenol pyruvate carboxylase specific activity	9 indicators: Presence of thorns in leaves (*); Presence of thorns in fruit crowns (*); Number of stomata per mm^2; Diameter of leaf vascular tissue; Thickness of the leaf aquiferous parenchyma; Thickness of the leaf photosynthetic parenchyma; Transpiration rate; Other aldehyde content; Content of free phenolics	10 indicators: Number of slips; Number of suckers; Water use efficiency; Internal leaf CO_2 concentration; Total chlorophyll concentration; Chlorophyll a concentration; Chlorophyll b concentration; Protein content; Phenylalanine ammonia-lyase activity; Phenylalanine ammonia-lyase specific activity	
Dwarf	13 indicators: Number of crowns in the fruit; Fruit content of vitamin C; Fruit acidity; Presence of thorns in leaves (*); Shape of fruit eyes (*); Presence of thorns in fruit crowns (*); Stoma diameter; Transpiration rate; Other aldehyde content; Content of cell wall-linked phenolics; Content of free phenolics; Phosphoenol pyruvate carboxylase activity; Phosphoenol pyruvate carboxylase specific activity	18 indicators: Plant height; Peduncle diameter; Fruit mass with crown; Number of eyes in the fruit; Fruit height; Fruit diameter; Plant generation cycle; Number of stomata per mm^2; Diameter of leaf vascular tissue; Thickness of the leaf aquiferous parenchyma; Thickness of the leaf photosynthetic parenchyma; Water use efficiency; Total chlorophyll concentration; Chlorophyll a concentration; Chlorophyll b concentration; Total content of phenolics; Superoxide dismutase activity; Superoxide dismutase specific activity	9 indicators: Number of shoots; Number of slips; Number of suckers; Photosynthetic rate; Internal leaf CO_2 concentration; Malondialdehyde content; Protein content; Phenylalanine ammonia-lyase activity; Phenylalanine ammonia-lyase specific activity	4 indicators: Leaf color from greenish with red zones to greenish (*); Plant architecture from lightly wide to compact (*); Fruit color from red-orange to yellow-green (*); Fruit shape from tonel to cylindrical-block (*)

which genes are involved in the morphological and physiological changes that were observed in this study. This paper differs from our previous one [43] largely in the addition of a large amount of phenotypic data. The additional data make it less likely that AFLP comparisons of the wild-type line and the two mutants will lead to tagging of the genes for thorn production and dwarf stature. On the other hand, AFLP-based gene tagging will require sexual hybridization between the two mutants. Due to the well documented pineapple self-incompatibility, we do not expect that the mutants set seed after pollination.

In *Ananas comosus* (L.) Merr., the self-incompatibility is brought about by inhibition of pollen tube growth in the upper third of the style [70,71]. It is gametophytically controlled by a single locus with multiple alleles [72]. The self-rejection reaction is variable in intensity and generally stronger in the cultivated varieties, which is probably a result of the domestication process and selection for seedless fruits [73]. Therefore, other strategies are in progress in our laboratory such as the linkage disequilibrium-based association mapping according to [74] to identify the genes for thorn production and dwarf stature. Studies about the sterility ratios of the new somaclonal variants, as well as, possibilities for somatic cell fusion are also being carried out.

4. Conclusions

We report here the identification of an agriculturally useful mutant (P3R5), with less thorny leaves and there-

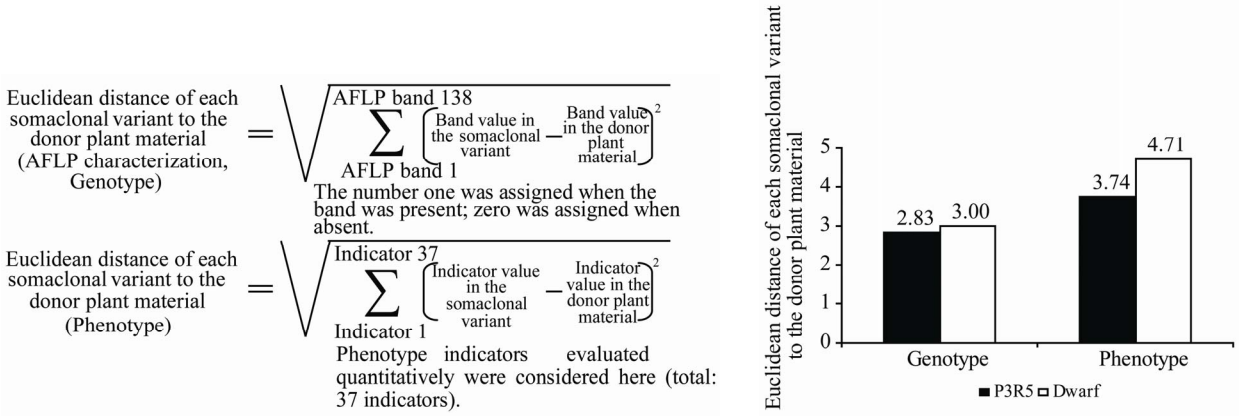

Figure 2. Euclidean distances of each somaclonal variant to the donor plant material (cv. Red Spanish Pinar). (a) Formulae; (b) Euclidean distances recorded.

fore, easier to manage in the field. Another mutant with ornamental value (Dwarf) was also obtained. Taking into consideration the phenotype, somaclonal variant Dwarf is more different from the donor plant than P3R5. The AFLP characterization supports these phenotype differences at genome level [43,44]. As far as we know, this is the first report of a comprehensive analysis of pineapple somaclonal variants.

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