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Cytological Analyses in 'Niagara Branca' Grape and in Its Somatic Mutant 'Niagara Rosada'

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Abstract

An investigation in the microsporogenesis of two cultivars of grapes (*Vitis* L.), were carried out on 'Niagara Branca' and on its somatic mutant 'Niagara Rosada', aiming at ascertaining the normality of the microsporogenesis. Some possible changes have been checked on chromosome number in somatic cells, pollen size and fertility, stomatal size and frequency, which related to the somatic mutant 'Niagara Rosada', thus increasing the information already obtained regarding 'Niagara' grapevines. The microsporogenesis occurred normally in both cultivars and cytomixis observed only among pachytene cells. A low percentage of precocious chromosome segregation was noticed in metaphase I and II, as well as a very low percentage of laggard and stickiness chromosomes in anaphase I and II. Less than 1% of tetrads showed microspore alterations. The mean values of the meiotic index and pollen grain fertility were high. However, no association were observed between the occurrences of chromosome abnormalities and the variation in the anther number (five to seven) seen among flower buds. The cultivars showed the same chromosome number, n=19 at diakinesis and $2n$=38 in somatic cells, and did not differ in regards to stomatal size and frequency. It seems that the difference between the cultivars lied at gene level. Due to the very low percentage of abnormalities and to high pollen grain fertility, the plants analyzed could be very well employed in breeding programs for the grapevines.

Keywords: chromosome abnormalities, microsporogenesis, pollen grain fertility, stomata size and frequency

Introduction

The 'Niagara' grape or fox grape (common grape) is native to the United States of America (USA) where it has been consumed as jam and juice. The cultivar 'Niagara Branca' was introduced in Brazil, in 1894 from Alabama (USA) while 'Niagara Rosada' arose as a somatic mutation of 'Niagara Branca' in 1933. Due to the attractive rosy color of the berries, 'Niagara Rosada' promptly captivated Brazilian consumer preference and taste (Maia, 2012). Both table grapes 'Niagara Branca' and 'Niagara Rosada' are considered easy-growing grapevines with a moderate tolerance to some diseases and despite the unusual 'slightly foxed' taste, they are also very much enjoyed. Although 'Niagara Branca' been had later been replaced by 'Niagara Rosada' on many farms, it is still grown on some few small farms mainly in the Brazilian Southern region (Silva *et al.*, 2006).

'Niagara' grapevine is a hybrid which resulted from an artificial crossing carried out in the USA at the end of 1868, in Niagara County, NY. Plants of *V. labrusca* 'Concord' were pollinated by 'Cassady' another hybrid (*V. labrusca* X *V. vinifera*), giving rise to the first 'Niagara' grapevines (Hedrick, 1919). Sometimes, however, 'Niagara' has been mentioned as a variety of *V. labrusca* (Fracaro *et al.*, 2004; Brum *et al.*, 2012, for instance). Despite the lack of a general agreement on this subject, Niagara cultivars have been studied through different approaches, such as genetics, physiology, disease resistance, soil adaptation, for instance, and much is known about their phenology (Tecchio *et al.*, 2011). Chromosome number in 'Niagara' was first reported as $2n$=38 by Takusagawa (1952).

Cytological works regarding grapes have predominantly focused on the detection of chromosome number and little is known about the genomic affinities of *Vitis* by means of chromosome comparative studies (Pierozzi, 2011). In addition, the few studies dealing with meiotic chromosome behavior in grapes are restricted to interspecific hybrids of *Vitis vinifera* with other wild species such as *V. rupestris* (Viljoen and Spies, 1995) and also restricted to 'vinifera' commercial varieties (Silva *et al.*, 2001; Direkçí and Buyukkartal, 2012), for instance and recently, Pereira *et al.* (2014) described in detail the meiosis of 'Touriga Nacional' after hot-water treatment. Despite the fact that 'Niagara Branca' and 'Niagara Rosada' cultivars being highly regarded table grapes, studies in microsporogenesis of 'Niagara' have not been carried out.

As part of chromosome research conducted with the grapevines cytological studies on the microsporogenesis were firstly carried out on 'Niagara Branca' and 'Niagara Rosada' with the purpose of ascertaining the microsporogenesis normality in both cultivars. Some possible changes checked, regarding chromosome number in somatic cells, pollen size and fertility, and stomatal size and frequency were possibly related to the somatic mutant 'Niagara Rosada', therefore enhancing the information already obtained about 'Niagara' grapevines.

Materials and methods

Plant material

'Niagara Branca' ('White Niagara') and 'Niagara Rosada' ('Rosy Niagara') are part of a small collection of different 'Niagara' cultivars which belong to the *Vitis* germplasm of IAC – Centro APTA de Frutas in Jundiaí (SP). Firstly, three plants of 'Niagara Branca' and 'Niagara Rosada' were chosen for the studies. Hydrogen cyanamid solution at 2.0% was applied to the 'Niagaras' grapevines bud break and also to promote uniformity in sprouting. Young inflorescences with flower buds at different stages of development been collected in two consecutive years, 2012 and 2013. The flower buds were fixed in Carnoy 3:1 solution (ethanol 99% and acetic acid, respectively) and stored in -20 °C until cytological preparations.

Cytological preparation

Two hundred buds of each cultivar were chosen at random, opened and the anther number scored. Anthers of some fixed young buds removed and squashed in 1.2% acetic carmine solution for cytological analyses carried out during the distinct meiotic phases from pachytene to pollen grains, and the percentage of chromosome abnormalities was scored. More than 700 pollen mother cells (PMCs) were evaluated in each meiotic phase for each cultivar. Chiasma average number per cell and per chromosome with the standard deviation was calculated using 100 cells at diakinesis phase. Meiotic index (MI) was also calculated. Silver impregnation according to Howell and Black (1980) was applied for nucleolus counting and also for a better visualization of the bivalent attached to the nucleolus at pachytene phase and 2,000 cells per cultivar were scored. Pollen grain fertility was estimated using Alexander A2 dye (Alexander, 1980) and considering cytoplasmic staining content. Full pollen grains, i.e., with cytoplasmic content stained were considered fertile while those that were unstained and empty were considered sterile and 4,000 pollen grains scored per cultivar. The size (area) of the fertile pollen grains was calculated, considering its ellipsoidal shape and 120 pollen grains of each plant were measured. The cellulose acetate technique (Medina, 1961) was applied for the obtainment of the stomatal size and the stomatal frequency (per mm^2). The colloidal was prepared according to the following: 1.2g cellulose acetate was diluted in 8mL of acetic acid was applied on small areas of the abaxial leaf surface employing thin painting brush, detached after 15 min, placed on a slide with 2 drops of distilled water, covered with cover glass and analyzed under microscope. Five expanded leaves from the intermediated region of each grapevine of each cultivar were collected in the morning and 125 stomata of each plant were measured and scored per leaf area (mm^2). Apart from chromosome counts in meiosis, the chromosomes of some diploid tapetal cells were counted. Photomicrographies were taken under 'Olympus Vanox' optical photomicroscope using a black and white TEMAX film ASA 125.

Results and discussion

Disturbances in chromosome behavior during meiosis are common in hybrid plants mainly in those with partial chromosome homology as observed by Viljoen and Spies (1995) in hybrid grapes obtained from artificial crossing of *V. vinifera* with *V. rupestris* and of *V. vinifera* with *V. rotundifolia*. However, despite the hybrid origin of 'Niagara', the microsporogenesis in both 'Niagara Branca' and 'Niagara Rosada' was normal (Fig. 1A to F; I and K). Cytological analyses of young buds showed only a few meiotic abnormalities (Fig. 1G to H and J; Tabs. 1 and 2) and up to 98% of the PMCs analyzed in 'Niagara Branca' as well as up to 90% in 'Niagara Rosada' presented normal behavior. Pachytene irregularities were not observed and less than 1% of pachytene cells displayed one bivalent with a short interstitial segment without pairing in 'Niagara Branca' (Fig. 2B).

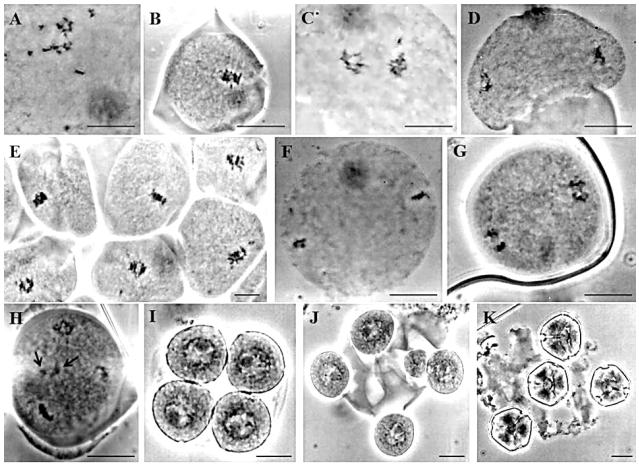

Fig. 1. Photomicrographies of some meiotic phases of 'Niagara Branca' (A to D, H and J) and 'Niagara Rosada' (E to G, I and K). A - diakinesis; B - metaphase I; C - anaphase I; D - telophase I; E- field with some metaphase I cells; F - metaphase II; G - anaphase with chromosome sticky II; H - 5 groups of chromosomes at telophase II; I - tetrad with four microspores; J - abnormal tetrad with 5 microspores of 'Niagara Branca'; K - pollen grains with full cytoplasm. Bar = 5μm; arrows = two small groups of chromosomes

Only one bivalent chromosome was observed attached to the nucleolus (Fig. 2A). The prevalence of one nucleolus with one bivalent associated could be considered as a characteristic inherited from 'labrusca' germplasm since 'Niagara' has 75% of *V. labrusca* germplasm (Maia, 2012). Variation in the number of bivalent associated to the nucleolus, from one to three, has been reported only in *V. vinifera* cultivars (Raj and Seethaiah, 1969, 1973, for

instance). These differences could be due to an intensive crossing that the vinifera varieties studied might have undergone over time. This may have led to asymmetric rearrangement or transposition events involving rDNA repeated sequences anchored in the nucleolus organizer region (NOR) to other chromosome pairs. Schubert (1984), for instance, reported the occurrence of asymmetric rearrangement, in commercial cultivars of onions, between NOR segments of one chromosome and a segment without NOR of some other non-homologous chromosome increasing the chromosome number carrying an active NOR. This hypothesis could also explain the higher nucleolus number observed in 'Brighton', four to six (Dorsey, 1914) when compared to both 'Niagara' studied. Since both 'Brighton' and 'Niagara' are interspecific hybrid of *V. vinifera* and *V. labrusca* it is also possible that the germplasm used in the crossing to give rise to 'Niagara' were more compatible/stable than those employed for the obtainment of 'Brighton'.

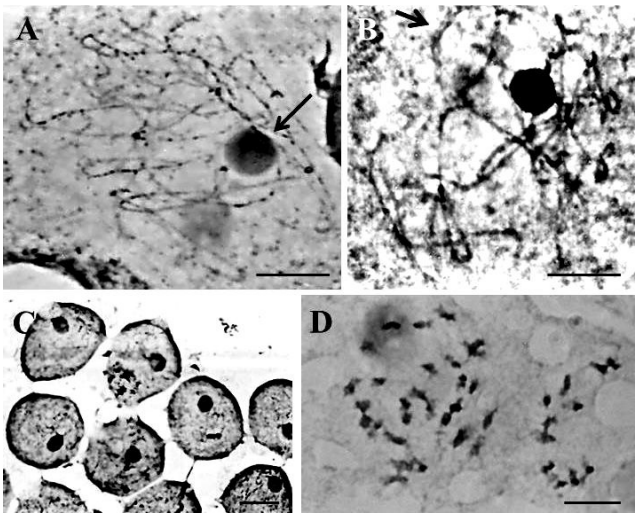

Fig. 2. Photomicrographies of 'Niagara Branca' (A and B) and 'Niagara Rosada' (C and D). A - pachytene showing one bivalent attached to the nucleolus (arrow); B - lack of pairing in a bivalent chromosome segment; C - Cytomixis among some pachytene microsporocytes; D - tapetal cell with diploid number of 38 chromosomes. Bar = 5 μm for C and D. Bar = 10 μm for A and B

Nineteen bivalents were observed at diakinesis and no chain or ring configurations which could suggest the presence of chromosome translocation were seen (Fig. 1 A). Chromosome stickiness was observed in 3.9% of PMCs at diakinesis only in 'Niagara Rosada' which also displayed a higher percentage of chromosome stickiness (nearly 9%) at metaphase I when compared to 'Niagara Branca' (Tab. 1). A low percentage of (a) precocious chromosome segregation at metaphase I and II; (b) stickiness and laggards chromosomes at anaphase I and II (Fig. 1G); and (c) three spindle at anaphase II were observed in both 'Niagara'. A very low percentage of cells with persistent nucleolus at metaphase I and cells with five nuclei at telophase II were seen only in 'Niagara Branca' (Tab. 1, Fig. 1 H).

The percentage of normal tetrads in both 'Niagara' was high (near 99%) and a low percentage of dyads (0.03%), triads (from 0.03% to 0.13%) and pentads (from 0.29% to 0.79%) (Tab. 2) were observed (Fig 1 I and J). Thus, the meiotic index value and percentage of pollen grain fertility mean values were high (over 93%) (Tab. 2). However, the percentage mean values of pollen fertility of 'Niagara Branca' were significantly higher than that of 'Niagara Rosada' (Tab. 2). Despite the hybrid origin of 'Niagara' the occurrence of a few chromosome abnormalities at a very low percentage in both cultivars was not strong enough to disturb pollen grain fertility, which was high. Significant percentages of chromosome irregularities may occur and have been reported in microsporogenesis of different varieties of *V. vinifera* (Silva *et al*., 2001; Direkçi and Buyukkartal, 2012) and most of these abnormalities were related to the presence of monovalent and multivalent chromosomes which ultimately reflected in the degree of pollen fertility. Pereira *et al*. (2014) reported degree of pollen fertility in *V. vinifera* 'Touruga Nacional' in plants treated with hot temperature.

Both cultivars did not differ at 5% level concerning chiasma mean values per cell (37.76±0.45 and 37.19±0.59 for 'Niagara Branca' and 'Niagara Rosada', respectively) and per chromosome (1.99±0.02 and 1.96±0.04 for 'Niagara Branca' and 'Niagara Rosada', respectively). In fact, the chiasma mean values for both 'Niagara' were higher when

Tab. 1. Number (N°) and percentage (%) of some meiotic abnormalities in pollen mother cells (PMCs) of 'Niagara Branca' (Bra) and 'Niagara Rosada' (Ro). Chrom = chromosome

	Branca		Rosada		Abnormalities	Branca	Rosada
Phase	Total	Normal	Total	Normal		N° (%)	N° (%)
Dia	730	730	756	726	Chromosome stickiness	0 (0.0)	30 (3.97)
Met I	761	747	844	744	Precocious chrom. segregation	11 (1.45)	03 (0.36)
					Chromosome stickiness	01 (0.13)	74 (8.77)
					II not aligned on the plate	0 (0.0)	23 (2.72)
					Persistent nucleolus at Met I	02 (0.26)	0 (0.0)
Ana I	733	724	717	700	Chromosome stickiness	09 (1.23)	14 (1.95)
					Laggard chromosomes	0 (0.0)	03 (0.42)
Met II	735	732	704	701	Precocious chrom. segregation	03 (0.41)	03 (0.43)
Ana II	729	718	744	728	Chromosome stickiness	07 (0.96)	12 (1.61)
					Laggard chromosomes	02 (0.27)	03 (0.40)
					Three spindle	02 (0.27)	01 (0.13)
Telo II	725	721	706	706	Five poles	04 (0,55)	0 (0.0)

Tab. 2. Mean values* for number of chiasma per cell and per chromosome, for percentage of normal and abnormal tetrads, meiotic index, pollen grain fertility, and for pollen grain size (μm²) for 'Niagara Branca' (Branca) and 'Niagara Rosada' (Rosada)

	Branca	Rosada
N° chiasma per cell	37.76±0.45^{ns}	37.19±0.59^{ns}
N° chiasma per chromosome	1.99±0.02^{ns}	1.96±0.04^{ns}
Tetrads:		
Normal (4 microspores)	99.08% ± 0.69^{ns} (2076)	99.65% ± 0.50^{ns} (2084)
Abnormal:		
Dyads (2 micorspores)	0% (0)	0.03% ± 0.1^{ns}
Triads (3 microspores)	0.13±0.28^{ns}	0.03% ± 0.1^{ns}
Pentads (5 microspore)	0.79% ± 0.79^{ns}	0.29% ± 0.39^{ns}
Meiotic Index	99.07% ± 0.69^{ns}	99.65% ± 0.50^{ns}
Pollen grain fertility	97.85% ± 0.78^{a}	93.45% ± 1.98^{b}
Pollen grain size	485.09μm²± 38.28^{ns}	498.87μm² ± 84.75^{ns}

*Mean values followed by different letters = significant at 5% after F-test. Ns=non-significant at 5% after F-test

compared to those obtained by Raj and Seethaiah (1969, 1973) in 'vinifera' hybrids, from 17.6 to 22.5 per cell and from 0.93 to 1.18 per chromosome. These differences could be a result of (a) intensive culture conditions imposed on the varieties of *V. vinifera*; and (b) significant disturbances in meiosis, as observed by those authors that led to a decrease in chiasma frequency.

The cultivars did not differ concerning pollen grain size (485.09 μm²± 38.28 and 498.87 μm² ± 84.75, for 'Niagara Branca' and 'Niagara Rosada', respectively) (Tab. 2) and no change was seen in pollen morphology which could be associated to the 'Niagara Rosada' somatic mutant. Dorsey (1914) reported variation in pollen sterility among some *V. labrusca* and *V. vinifera* hybrids which varied from 1% in 'Black Eagle' to 50% in 'Niagara' growing in Minnesota fields (USA). Besides the interaction between genotypes with climate and soil conditions, it is possible that the 'Niagara Branca' genotypes introduced in Brazil may have shared a parental germplasm with high affinities, leading to a more stable chromosome pairing which eventually reflected on the absence of monovalent chromosomes as well as on the chiasma formation and visualization.

The chromosome number counted in diploid tapetal cells was 2n=38 in both 'Niagara' cultivars (Fig. 2.D), the same number reported by Takusagawa (1952).

Cytomixis was observed in 'Niagara' pachytene cells (Fig. 2C). Cytomixis is defined as cytoplasmic bridge-building between two or more PMCs and, in some cases, there is migration of one or more chromosomes from one cell to another. The process is often related to the reduction of pollen fertility while some factors such as herbicides or pesticide treatment on crop culture can trigger the cytomixis. Laggard chromosomes and chromosome stickiness have also been documented in association with cytomixis in ramie (Pierozzi *et al*., 2008). A possible explanation for this association is that some genetic and also environmental factors might play a role in the occurrence of this relationship, as suggested by Sheidai *et al.* (2003). Although no chromosome or chromatin segment migration was observed through the cytomictic channels at pachytene stage in Niagara cultivars, this possibility was not disregarded.

'Niagara Branca' and 'Niagara Rosada' displayed anomocyte type of stomata (Najmadin *et al.* 2011) which were localized only on the abaxial leaf surface. Since stomatal frequency can vary among *V. vinifera* cultivars under the same climate and soil conditions (Monteiro *et al.*, 2013) it has been an interesting parameter for species or varieties characterization. In fact, information obtained through stomatal traits has also been very useful as a taxonomic tool for the separation of *V. vinifera* cultivars from some wild non-vinifera (non-cultivated) species (Swanepoel and Villiers, 1987). Considering the stomata size, both 'Niagara' displayed mean values close to those obtained for 16 cultivars of *V. vinifera* of Anatolian vineyards (Turkey) (Eris and Soylu, 1990) and for four other cultivars growing in Portugal (Monteiro *et al.*, 2013) and lower than those reported for *V. labrusca* and for some other cultivars of *V. vinifera* (Swanepoel and Villiers, 1987). The mean values for stomata frequency were close to those obtained for some 'vinifera' hybrids but lower than those for *V. labrusca* germplasm analyzed by Swanepoel and Villiers (1987). However, no significant variation concerning the mean values of the stomatal size (23.85 μm±1.09 length and 13.95 μm±0.70 width for 'Niagara Branca'; 23.99 μm±0.65 length and 15.68 μm±1.70 width for 'Niagara Rosada') and in regards to the frequency (200.42±6.27 and 191.79±5.42 for 'Niagara Branca' and 'Niagara Rosada', respectively) was observed (Tab. 3). Therefore, the stomata traits analyzed did not constitute an informative parameter toward 'Niagaras' differentiation.

Tab. 3. Percentage of stamen numbers in floral buds, stomata size and stomata frequency considered in 'Branca' and 'Rosada'

	Branca	Rosada
Floral buds with:		
5 anthers	76.17%±10.33^{ns}	65.49%±8.47^{ns}
6 anthers	23.83%±10.34^{ns}	34.08%±15.69^{ns}
7 anthers	0.00^{ns}	0.44%±1.79^{ns}
Stomata size:		
length	23.85μm±1.09^{ns}	23.99μm±0.65^{ns}
width	13.95μm±0.70^{ns}	15.68μm±1.70^{ns}
Stomata	200.42+6.27^{ns}	191.79+5.42^{ns}

The anther number per flower bud varied from five to seven (Tab. 3) and a prevalence of five anthers per floral buds was observed in both cultivars, 76.17%±10.33 in 'Niagara Branca' and 65.49%±8.47 in 'Niagara Rosada'. Buds with 7 anthers were only seen in 'Niagara Rosada' at a

very low percentage (0.44%±1.79). Variation in the anther number (from three to nine) among the bud flowers of some species and cultivars of grapes was first observed by Dorsey (1912) seeing that buds with five anthers were observed in more than 60% of the genotypes analyzed. It is the first time that differences in the number of anthers in flower buds of 'Niagara' grapes growing in Brazil were reported and the cause(s) of this variation may be due to a genetic source or caused by external factors such as temperature fluctuations during sprouting, water availability or chemical agents. The use of a diluted solution of hydrogen cyanamid has been a very common practice since it has been proved to be an efficient agent for dormancy bud break of temperate deciduous plants, such as grapes, which have been cultivated in tropical climates (Botelho *et al.*, 2002). Nevertheless, it is known that some types of herbicide or hormone-like products such as hydrogenated cyanamid for instance, when applied in high concentrations have phytotoxic effect and may cause disturbances in cell divisions (Soltys *et al.*, 2011), which may ultimately reflect in plant fruit or grain yielding. However, it was impossible to state with certainty if the occurrence of cytomixis and/or the variation in the number of anther in flower buds observed in 'Niagara Branca' and 'Niagara Rosada' could be attributed to the utilization of this chemical agent. Nevertheless, this subject constitutes an open question which calls for further studies.

Finally, in grapes, the color of the berry skins**,** in grapes**,** is determined by the accumulation of anthocyanins whose biosynthesis is regulated by *VvmybA1* and *VvmybA2* genes (Mitani *et al.*, 2009). In *V. vinifera* the insertion of a retrotransposon *Gret 1* upstream *VvmybA1* or the occurrence of a single-nucleotide polymorphism mutation in *VvMybA2*, is enough to disrupt the gene expression and result in berries with white skin (Fournier-Level *et al.*, 2009). It is also known that red and rosy berry colors can arise through mutations in *Gret 1* which leads to the reduction of *VvMybA1* repression at different levels (Pelsy, 2010). Knowing that the retroelement *Gret 1* appeared only in European *V. vinifera* and probably in the *V. sylvestris* (Mitani *et al.*, 2009) and considering that hybrid 'Niagara' has 'vinifera' germplasm, it seems more likely that the somatic mutation was in fact a reversion in berry color from white ('Niagara Branca') to rosy ('Niagara Rosada'). This could be due to an instability in the insertion of this retrotransposon inherited from *V. vinifera* by elimination or impairing during the cell divisions at the bud burst stage, thus restoring the anthocyanins biosynthesis and consequently the 'Niagara' skin color.

Interestingly, the instability in berry color of 'Niagara' has been noticed in the grapevine collection and also in field production. It is possible to see, on the same cane, bunches of grapes with white berries along with bunches with rosy berries. This unexpected variability has led some researchers to regard this as a case of bud sports. The depicted mutation, which gave rise to 'Niagara Rosada', did not affect chromosome number which was the same in both cultivars.

Since grapevines are perennial crops they are constantly under cultivation pressure such as pruning, pesticide and herbicides exposure, and other chemical agents, besides unfavorable climate conditions such as the 'Niagara' crop, therefore the changes or instabilities of berry color, could as a matter of fact, very well be expected.

Conclusion

The phenotypic difference between 'Niagara Branca' and its somatic mutant 'Niagara Rosada' was not related to chromosome number variation, ploidy level or disturbance in the microsporogenesis since this process was normal and the very low percentage of chromosome abnormalities observed did not affect neither the meiotic index nor the pollen grain fertility, which were high. The spontaneous somatic mutant seems to be a consequence of an unstable gene expression related to anthocyanins biosynthesis. Due to the very low percentage of abnormalities and to high pollen fertility, the plants analyzed may well be employed in breeding programs for the grapevines.

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