Rev. Brasil. Genet. 14, 2, 413-423 (1991)
(Brazil. J. Genetics)

BIOCHEMICAL CHARACTERIZATION OF ANTHERS FROM MALE-STERILE COFFEE PLANTS

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ABSTRACT

Two male-sterile coffee trees of *Coffea arabica*, an accession from Ethiopia, C2154-2 and the cultivar Blumor (Blue Mountain x Timor Hybrid), C2018-8, as well as a fertile variety belonging to the same species, were submitted to daily harvesting of flower anthers for a period of five days before flowering. On the sixth day, when blossoming occurred, the anthers were harvested four times from each coffee plant.

On the basis of estimates of soluble carbohydrate, sucrose, protein and free amino acid contents in the anthers, it was concluded that the metabolic activities of the anthers obtained from the fertile plant were substantially higher than those of the anthers from the male-sterile accessions. The amino acid composition estimated for the material collected three days before flowering varied appreciably from that detected in the material from the first harvesting on the sixth day. In this respect, by flowering time serine was found to have accumulated to the greatest extent in the fertile variety and in the male-sterile C2154-2, and to have decreased in C2018-8. In addition, serine was found to be the amino acid that always accumulated to the largest extent in the anthers, regardless of the coffee plant variety. Proline accumulation followed the same pattern and was second to serine in terms of amount accumulated, also decreasing drastically in C2018-8 at flowering time. A similar behavior was observed for glutamic acid and ammonium nitrogen.

INTRODUCTION

In 1984, Mazzafera *et al.* reported the occurrence of a type of male sterility in coffee in which no pollen production occurred in the anthers of *Coffea arabica*

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accessions from Ethiopia and in a cultivar of the same species denoted Blumor (Blue Mountain x Timor hybrid).

To study the inheritance of this trait and to transfer it to commercial coffee lines for use in the production of hybrid seed and in selection programs, a series of crosses were started with other *C. arabica* coffee plants and the results obtained led Mazzafera *et al.* (1989) to conclude that this type of sterility may very probably be conferred by recessive nuclear genes. According to Mascarenhas (1975) and Frankel and Galun (1977), the cause of this type of male-sterility may be due to certain disorders in the necessary physiological relationship between tapetum cells and developing microspores. Mascarenhas (1975) also emphasized the existence of circumstancial evidence, mainly of a cytochemical and ultrastructural nature, that relates the tapetum to the nutrition of developing microspores. Differences in the levels of certain substances during microspore development may characterize the biochemical communication between microsporocytes and tapetum in normal and sterile anthers (Frankel and Galun, 1977).

Cytological observations by Mazzafera *et al.* (1989) showed that tapetum and microsporocyte degeneration occurs after mitosis. However, no biochemical studies have been conducted on these coffee plants to characterize the physiological relationship between microspore and tapetum.

Thus, using a fertile coffee plant as standard, in the present study we report on the monitoring of anther development (before flowering and on the day of flowering) of male-sterile coffee plants by the determination of the contents of total soluble sugars, soluble proteins, sucrose and amino acids.

MATERIALS AND METHODS

Anthers were removed from flowers of the coffee cultivar Mundo Novo CP388-17, of the accession from Ethiopia C2154-2, and of the cultivar Blumor C2018-8. The latter two coffee plants are male-sterile and were planted in production trial no. 228 in the experimental field of the Genetics Section, Agronomy Institute of Campinas. Flowers were collected during the five days preceding blossoming. On the fifth day, some of the collected flowers were placed on Petri dishes containing cottonwool moistened with water. On the sixth day, when the flowers opened on the Petri dishes, the anthers were collected four times at regular 3.5-hour intervals, starting at 10:30 hours. Pollen production was observed as early as during the first collection on the sixth day in the anthers of the fertile plant.

Immediately after removal from the flowers, the anthers were stored in a freezer (-18°C) and later lyophilized. Lyophilized anthers were immersed in a 12:5:3 methanol:chloroform:water solution (Bielesk and Turner, 1966) for one week at 5°C.

The addition of water and chloroform after this period led to the formation of an ethanol-aqueous phase and a chloroform phase, and the extract was left to stand at 5°C for three additional days. Aliquots of the methanol-aqueous phase were dried on a waterbath at 35°C, resuspended in distilled water and used to measure soluble sugar, sucrose and free amino acid contents by the methods of Dubois *et al.* (1956), Van Handell (1968) and Cocking and Yemm (1954), respectively. Samples from the collections made on the third day before flowering and from the first collection on the flowering day were also analyzed for amino acid content using an automatic Aminochrom II analyzer.

The anthers recovered from the extracting solution were quickly washed with methanol and extracted three times by soaking in 0.1 N NaOH. Soluble protein content was measured in the pooled extracts by the method of Bradford (1976).

RESULTS

Table I shows the levels of protein and free amino acids detected in the processed anthers. It can be seen that protein content was intensely decreased in Mundo Novo from the first to the last sampling (about 9.5 times), indicating intense metabolic activity. This statement is based on data on free amino acid content which, contrary to protein content, was increased ($r = -0.85$, $P < 0.05$).

A similar occurrence was noted for the male-sterile plant C2154-2, whose protein content was reduced by half. The inverse correlation, however, was not as intense ($r = -0.68$, $P < 0.05$) as for Mundo Novo. C2018-8 also showed nearly 50% reduction in protein content, but there was no correlation with amino acid content ($r = -0.16$, not significant), which was unchanged throughout the study.

Table II presents soluble sugar and sucrose levels. Soluble sugar content was stable in Mundo Novo, whereas sucrose was increased almost three-fold. A reduction in soluble sugars occurred in C2154-2 and C2018-8, with an increase in sucrose that was not as high as for Mundo Novo. Thus, the increase in sucrose in relation to reducing sugars in the male-sterile coffee plants was small and much lower than in Mundo Novo. At the first sampling, sucrose was 11% and 5% of the soluble sugar content in C2154-2 and C2018-8, respectively, and increased to 24% and 14% respectively at the last sampling. In Mundo Novo, sucrose content changed from 12% to 36%, reaching 40% on the occasion of the first two samplings on the flowering day. These data also indicate greater metabolic activity in the anthers of the fertile cultivar.

Quantitative amino acid analyses performed on the anthers of flowers collected three days before flowering (3) and at the beginning of flowering (6A) showed a wide variation for certain amino acids, although a certain similarity was observed between Mundo Novo and C2154-2 on the third day before flowering (Table III).

Table I - Soluble protein and free amino acid contents in anthers of male-sterile (C2154 and C2018-8) and fertile (Mundo Novo) coffee plants before and during blossoming.

Proteins[a,b] (mg/g d.m.)	Sampling times[c] 1	2	3	4	5	6A	6B	6C	6D
Mundo Novo	79.03	71.83	55.58	27.58	39.47	19.25	16.97	10.18	8.68
	(0.80)	(4.36)	(0.64)	(4.48)	(1.63)	(1.81)	(6.70)	(5.91)	(1.30)
C2154-2	56.54	67.59	51.56	34.56	33.23	29.01	35.91	42.31	35.26
	(2.81)	(1.75)	(5.95)	(0.30)	(1.12)	(0.81)	(6.46)	(0.50)	(8.08)
C2018-8	114.24	90.89	103.66	67.79	68.08	64.28	58.56	32.07	56.41
	(24.06)	(17.05)	(1.47)	(3.83)	(6.14)	(2.93)	(7.23)	(6.80)	(5.98)
Amino acids (μmol/gd.m.)									
Mundo Novo	138.01	158.03	158.56	215.87	172.19	245.99	255.62	183.36	237.14
	(8.27)	(4.86)	(13.59)	(18.97)	(18.44)	(3.54)	(4.75)	(1.20)	(2.48)
C2154-2	52.76	46.13	67.47	64.04	69.77	162.19	132.77	151.25	136.73
	(6.20)	(0.10)	(5.28)	(4.68)	(3.25)	(7.69)	(0.05)	(7.71)	(2.38)
C2018-8	161.10	78.71	106.17	117.41	103.86	122.71	161.59	128.58	157.39
	(13.41)	(0.70)	(0.52)	(2.99)	(5.57)	(2.08)	(2.28)	(0.40)	(0.70)

[a] Mean of three replications.
[b] The values in parentheses are the standard deviations.
[c] Time 1 corresponds to the first sampling performed five days before blossoming, which occurred at time 6. A, B, C and D for time 6 correspond to anther samplings performed at 3.5-hour intervals starting (A) at 10:00 hours.
d.m. = dry matter.

When C2154-2 was compared with Mundo Novo, anthers from flowers collected three days before flowering had similar amino acid levels except for proline and serine whose levels were higher in Mundo Novo. When pre-flowering values were compared to the values obtained on the flowering day, leucine, isoleucine, valine and glycine increased twice as much in C2154-2 than in Mundo Novo. In contrast, proline and aspartic acid levels were more increased in the fertile coffee plant. Glutamic acid and serine levels increased in both coffee plants, but the increase observed in the male-sterile plant in relation to the content detected on the third day was almost double that observed in Mundo Novo. The variations in the other amino acids were few and proportionally similar in the two coffee plants.

Table II - Soluble sugar and sucrose content in anthers of male-sterile (C2154-2 and C2018-8) and fertile (Mundo Novo) coffee plants before and during blossoming.

Soluble sugars[a,b] (mg/g d.m.)	Sampling times[d]								
	1	2	3	4	5	6A	6B	6C	6D
Mundo Novo	73.86 (6.15)	75.49 (0.78)	68.38 (2.35)	122.03 (1.46)	80.63 (1.99)	68.35 (3.32)	70.01 (6.74)	79.56 (6.73)	77.81 (1.65)
C2154-2	110.67 (1.68)	94.05 (1.90)	113.59 (4.29)	97.04 (0.23)	72.86 (0.30)	98.96 (0.51)	89.39 (0.20)	77.96 (0.95)	80.26 (3.60)
C2018-8	183.21 (7.85)	120.85 (26.35)	123.06 (3.85)	131.92 (5.78)	103.84 (7.94)	97.02 (0.23)	110.29 (1.85)	95.77 (0.47)	103.86 (1.99)
Sucrose (mg/gd.m.)									
Mundo Novo	9.38 (0.24)	7.25 (0.07)	6.44 (0.14)	22.90 (0.63)	9.14 (0.40)	31.73 (1.67)	30.92 (0.47)	29.80 (0.41)	27.83 (0.16)
C2154-2	12.04 (0.18)	7.09 (0.13)	10.38 (0.45)	15.18 (1.36)	4.62 (0.13)	19.37 (0.45)	19.89 (0.93)	17.61 (1.73)	19.51 (1.44)
C2018-8	9.21 (0.19)	7.60 (0.37)	9.68 (1.10)	17.68 (1.38)	13.03 (1.17)	16.73 (0.74)	19.07 (0.03)	18.98 (1.78)	14.67 (0.11)

See footnotes of Table I.

Table III - Free amino acid composition of anthers from male-sterile (C2154-2 and C2018-8) and fertile (Mundo Novo) coffee plants collected three days before (3) and at the beginning of the day of blossoming (6A).

Amino acids[a,b,c]	Mundo Novo		C2154-2		C2018-8	
(μ mol/g m.s.)	3	6A	3	6A	3	6A
Arginine	4.67	5.42	8.83	10.25	6.09	3.25
Histidine	0.91	10.80	ND	15.98	4.51	5.52
Lysine	5.85	4.15	8.29	11.69	10.66	4.83
Phenylalanine	4.88	7.99	3.85	6.31	8.22	3.93
Tyrosine	2.09	6.93	2.21	7.33	4.18	2.67
Leucine	11.42	18.58	4.89	44.43	20.57	15.74
Isoleucine	5.46	29.75	4.38	65.03	13.90	21.62
Valine	9.02	47.03	8.24	82.41	23.86	28.06
Alanine	14.08	21.68	9.37	33.78	46.96	11.55
Glycine	4.02	5.14	2.73	9.29	7.70	3.08
Proline	61.05	104.84	18.71	40.48	67.65	12.30
Glutamic acid	33.15	89.35	28.75	128.76	95.18	34.90
Serine	225.79	786.18	139.46	861.93	523.84	314.31
Aspartic acid	16.51	46.84	12.11	18.49	47.05	13.34
Methionine	1.11	ND	ND	ND	2.13	ND
Cysteine	1.72	3.26	3.35	8.44	4.40	4.40
NH_4	30.09	53.59	45.59	96.47	75.56	44.95
Total	401.73	1187.94	255.17	1344.60	886.90	479.50

[a] ND, Not detected.
[b] Ammonia was not included in the amino acid total.
[c] Proline was detected at 440 nm.

The variations in amino acid contents observed in C2018-8 differed considerably from those observed in C2154-2. In the analyses of anthers from flowers collected before flowering, C2018-8 showed slightly higher values of certain amino acids which, contrary to what occurred with Mundo Novo and C21542, were decreased on the flowering day. This was the case for glycine, lysine, phenylalanine and tyrosine. Since phenylalanine and tyrosine are formed in the shikimic acid pathway which initiates with pyruvic acid and erythrose-4-phosphate (Haslam, 1974), their reduced levels may indicate a lower respiratory rate. This hypothesis agrees with the small reduction in protein content and with the reduced sucrose content in C2018-8.

In contrast to C2154-2, C2018-8 showed few changes in leucine, isoleucine and valine levels. The values of proline, glutamic acid and aspartic acid obtained at first analysis for C2018-8 were much higher than those obtained for C2154-2 and for Mundo Novo (with the exception of proline). The levels of all three amino acids were reduced on the sixth day of sampling.

Since aspartic acid and glutamic acid are the first amino acids involved in ammonia uptake in the nitrogen metabolism of plants for the synthesis of other amino acids (Ting, 1982), the variations observed here for this ion and these amino acids are coherent.

The differences in the total amino acid contents determined with an automatic analyzer compared to those obtained by the colorimetric method of Cocking and Yemm (1954) are due to the fact that leucine was used as a standard in the colorimetric method. While leucine has a molecular weight of 131, serine, which was the amino acid detected in largest amounts with the analyzer (± 60% of total amino acid content), has a molecular weight of 105, a fact that leads to an underestimate by the method of Cocking and Yemm (1954).

In the analysis performed with the automatic amino acid analyzer, a substance was detected between lysine and phenylalanine but it could not be identified.

Unknown substances were also detected by Macrae (1985) and McDonald and Macrae (1985) in amino acid analyses performed on coffee seeds. However, because of their unknown nature, it was not possible to quantify them.

DISCUSSION

Few biochemical comparisons of developing fertile microspores with microspores of genetically male-sterile plants have been conducted. Most of these studies were made with plants with cytoplasmic male sterility, an understandable fact in view of the greater interest in this type of sterility, of the easy maintenance and transfer of this trait to other lines, and also because of the difficulty in obtaining a 100% male-sterile stand in an economically viable manner (Frankel and Galun, 1977).

In studies on cytoplasmic male-sterile plants, proline content was usually found to be reduced in the anthers (Khoo and Stinson, 1957; Brooks, 1962; Alam and Sandal, 1972; Tsvestikova, 1979). According to Stanley (1971, cited by Johri *et al.*, 1977), this suggests that proline may be essential for pollen germination. Daskek and Hardwood (1974) observed that high levels of proline accumulated in pollen grains of *Lilium longiflorum* cv. Ace and that these levels were drastically reduced during pollen tube growth. These authors concluded that proline may be a readily available source of energy.

Stewart *et al.* (1966), studying the relationship between proline accumulation and sugar content in plants submitted to water stress, concluded that proline accumulation depends on the carbohydrate supply. However, none of the studied in which proline content was found to be reduced in male-sterile anthers related this fact to sugar content.

Tsvetikova (1979) observed an increased accumulation of alanine in two types of cytoplasmic male-sterile corn plants, Texas and Maldavian, when compared with fertile lines. Excess ammonia produced by the use of protein in respiration and when carbohydrates were present in insufficient amounts was neutralized by asparagine formation in type M anthers and by alanine formation in type T anthers. Anthers from male-sterile plants, and those of type T in particular, had a lower proline content.

In an analysis of work by Fukusawa (1962) and by Izhar and Frankel (1973), Frankel and Galun (1977) concluded that the free amino acid balance in anthers may be subjected to a series of factors such as protein degradation and synthesis, and the alteration in the amino acid profile may have an effect both on metabolic and catabolic processes. Thus, the increased asparagine content observed by Izhar and Frankel (1973) in *Petunia* anthers with cytoplasmic sterility may be a consequence of tissue degradation or some blockage in the metabolic pathway between asparagine and ornithine in the tricarboxylic acid cycle, as suggested by Fukusawa (1962).

According to the present results, the anthers of genetically male-sterile coffee plants show a biochemical behavior differing from that of Novo Mundo. However, the two male-sterile coffee lineages studied presented certain differences, especially in terms of amino acid composition. In principle, these differences may be explained by the distinct genotypic nature of the two materials, suggesting that, rather than using Mundo Novo as standard, the ideal experiment would have been to compare the male-sterile plants with sister plants. However, since the main objective of the present study was to monitor the biochemical variations in the anthers, Mundo Novo, a very productive cultivar, was considered to be a good standard.

Depending on the genotype, proline both increased (C2154-2) and decreased (C2018-8) in anthers from male-sterile plants on the flowering day, thus preventing any inferences about whether this amino acid is essential for coffee pollen germination. Also, the sucrose levels on the day when the quantitative amino acid analyses were performed showed that sugars were available in C1028-8, in which proline content was reduced. Probably, the relationship established by Stewart *et al.* (1966) between proline and carbohydrates is applicable only under conditions of water stress. In any case, after serine, whose metabolic implication in the present study is unknown, proline was the amino acid detected in largest amounts in fertile coffee anthers, thus together with serine attracting more interest in later studies.

The data obtained here for the variation in ammonia (Table III) and protein (Table I) levels agree with the proposition of Tsvetikova (1979) that the increase in ammonia may be due to greater protein degradation through respiration. In C2154-2 and Mundo Novo, released ammonia may be mainly incorporated into serine and glutamic acid.

The greater proportion of sucrose in the anthers of the fertile plant may have been due to the importation of this sugar from the leaves, since the flowering of coffee trees is known to require a large supply of carbohydrates (Melotto, 1987). This probably occurred due to a high respiratory rate, which produced the energy needed for pollen grain formation. Since in the anthers of C2018-8 and C2154-2 there is no pollen formation and there is tapetum degeneration (Mazzafera *et al*., 1989), with a consequent interruption of the nutritional role of this structure (Mascarenhas, 1975), the energy expenditure occurring in male-sterile plants may be much lower.

On this basis, we conclude that the male-sterile coffee lines C2018-8 and C2154-2 are similar in terms of the metabolic behavior of their anthers but differ in this respect from the fertile coffee plant Mundo Novo. The marked differences in amino acid composition observed between the male-sterile plants and Mundo Novo can only be explained by the fact that these are different cultivars although they all belong to the species *C. arabica*.

ACKNOWLEDGMENTS

Publication supported by FAPESP.

RESUMO

Dois cafeeiros portadores de macho-esterilidade gênica, a introdução C2154-2 da Etiópia e o cultivar Blumor (Blue Mountain x Híbrido do Timor) C2018-8, e um cafeeiro fértil, todos pertencentes à espécie *Coffea arabica*, tiveram anteras coletadas de suas flôres a partir de cinco dias antes do florescimento e por quatro ocasiões neste dia. Análises do teor de açúcares solúveis, sacarose, proteínas e aminoácidos livres, indicaram que nas anteras do cafeeiro fértil havia uma atividade metabólica mais intensa no florescimento do que nos macho-estéreis. Análises da composição de aminoácidos em anteras coletadas três dias antes e no dia do florescimento indicaram variações acentuadas. Serina sempre foi o aminoácido em maior quantidade nas anteras dos três cafeeiros, tendo aumentado por ocasião do florescimento no cafeeiro fértil e em C2154-2, e diminuído em C2018-8. Prolina era o segundo aminoácido em quantidade nas anteras dos três cafeeiros três dias antes do florescimento, tendo aumentado nessa

época no cafeeiro fértil e no C2154-2, e diminuído drasticamente em C2018-8. Comportamento semelhante ocorreu com ácido glutâmico e amônia.

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(Received November 25, 1989)