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Genetic variation of phytochemical compounds in progenies of *Ilex paraguariensis* St. Hil.

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ABSTRACT - *Mate (*Ilex paraguariensis *St. Hil) contains phytochemical compounds capable of preventing a number of health problems. Knowledge on the genetic contribution to the variability in these compounds can help to obtain mate progenies with higher levels thereof in breeding programs. The composition of triterpene saponins, methylxanthines, chlorogenic acid and the antioxidant activity of eight mate progenies were evaluated. Significant differences among progenies were verified in contents of triterpene saponins (0.003-0.080%), caffeine (0.226-1.377%), theobromine (0.176-0.831%), and chlorogenic acid (1.344-2.031%) and in antioxidant activity (31.251-51.406%). The contents of theobromine were found to be negatively correlated with saponins and caffeine, and caffeine with chlorogenic acid, while theobromine was positively correlated with chlorogenic acid. The heritability values for saponins (75.09%), caffeine (75.19%), theobromine (66.87%), chlorogenic acid (52.86%) and antioxidant activity (67.75%) indicate the possibility of genetic gain in selection for these traits.*

Key words: *Aquifoliaceae*, triterpene saponins, methylxanthines, chlorogenic acid, antioxidant activity.

INTRODUCTION

Mate (*Ilex paraguariensis* St. Hil - *Aquifoliaceae*) is the raw material for Mate or Chimarrão and Tereré, very popular beverages in South America (Reginatto et al. 1999). The largest producers of mate are Brazil, Argentina and Paraguay, where the crop has a high cultural, social and economic importance (Costa et al. 2005).

The main compounds found in mate are saponins (derivatives of ursolic and oleanolic acid), methylxanthines (caffeine, theobromine and theophylline) and phenolic compounds (chlorogenic acid, caffeic acid and 3,4, 3,5 and 4,5-dicaffeoylquinic acid), which contribute to the antioxidant, hepatoprotective activity (Campos et al. 1996), stimulating the heart and central nervous system (Da Croce 2002), hypocholesterolemic and diuretic (Gnoatto et al. 2005), attributed to mate.

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Several factors can influence the levels of chemical compounds in mate, as for example intra-species variation (Coelho et al. 2001), harvest time (Reginatto et al. 1999, Coelho et al. 2001, Schubert et al. 2006), origin, and genetic variability (Scherer et al. 2002, Costa et al. 2005, Cardozo Junior et al. 2007). Little information is available on the genetic components of this variability. A quantification of the genetic variation of these compounds is important for the development of breeding strategies to select progenies with higher levels of these constituents.

Genetic improvement of mate was initiated in Brazil in 1986 by the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI), and in 1995 by the Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), in the state of Paraná, which further the evaluation of half-sib progenies, providing base populations for genetic improvement.

Studies on improvement generally focus on mass production and disease resistance (Costa et al. 2005). However, due to the chemical composition, the levels of these compounds should also be considered in improvement programs. Estimates of genetic parameters can be used by breeders for a more detailed knowledge on the genetic potential of plants to be selected and recombined to improve the chemical characteristics of interest in this species. Efforts have been made to assess the chemical composition of *I. paraguariensis* in breeding programs (Costa et al. 2005, Cardozo Junior et al. 2007). The purpose of this study was to evaluate the variation in levels of triterpene saponins, caffeine, theobromine, chlorogenic acid and antioxidant activity in eight selected mate progenies. The genetic parameters, heritability and the correlations of each of these compounds were also analyzed.

MATERIAL AND METHODS

Plant Material

Mate samples were selected in a progeny trial of a genetic improvement program conducted on the experimental unit of Embrapa Florestas, on the Fazenda Vila Nova from Ervateira Bitumirim in Ivaí - PR (lat 25 ° 01 'S, long 50 ° 48' W, alt 650-750 m). The soil is dystrophic clayey (72% clay) dark-red Latosol (4% slope). The climate is mesothermic humid subtropical (Cfb), with hot summers and a tendency of rain concentration, without a clearly defined dry season and few frosts. The mean annual temperature is 17 - 18 °C.

The progenies were planted in March 1997 in an experimental design of randomized blocks with 10 replications. The progenies were planted at a spacing of 3 m between rows, 2 m between trees and 3 m between blocks. The plants were pruned once at an age of about two years and again about two years later.

From a total of 51 progenies, eight were selected in preliminary analysis of the contents of phenolic compounds and methylxanthines (Cardozo Junior et al. 2007). These data were used in a cluster analysis, using Mahalanobis generalized distance and the UPGMA clustering method (Unweighted Pair–Group Method with Arithmetic Averages), as described by Cruz and Carneiro (2003), using the GENES program (Cruz 2006). Based on this result one progeny of each group was chosen in order to obtain the most divergent progenies possible.

Branches with leaves were collected in July 2007 in the morning, in three blocks, from the median/mid part of the trees on the east side. The leaves were immersed in boiling water (100 °C) for 5 seconds (enzyme inactivation) and heated in a forced-air oven at 45 °C for 48 hours. The samples were then ground, labelled and placed in amber glass, sealed and stored under refrigeration.

Extraction of Saponin

Fifteen grams of the samples were boiled for 15 minutes with water (plant: solvent 1.5:13 m/v). The extracted solution was vacuum-filtered through filter paper (Whatman) and the final volume adjusted to 100 mL. The aqueous extract of *I. paraguariensis* was treated with 33 mL HCl (Chloridric acid) PA resulting in a solution at a concentration of 4 mol L maintained at reflux for 1 hour. The saponins were extracted with chloroform (50 mL x 4). The chloroform fraction was evaporated at room temperature and the residue reconstituted with 50 mL acetonitrile. This solution was filtered through a 0.45 μm membrane (Millipore, HVHP) and analyzed by high performance liquid chromatography (HPLC), according to a methodology adapted from Gnoatto et al. (2005). The entire extraction process was monitored by thin-layer chromatography (TLC) and compared with the ursolic acid standard (Sigma).

Methylxanthine extraction

The reflux system was used, where 1.0 g sample was immersed in 70% methanol (5 x 50 mL) for 20 minutes. The extract was treated with Carrez reagent (protein precipitation) at a ratio of 2 mL reagent A : 2 mL reagent B, and vacuum filtered (Whatman No 4), according to the modified method of Clifford and Ramirez-Martinez (1990).

Extraction of chlorogenic acid

One gram of sample was extracted by maceration with 70% methanol (5x10 mL), obtaining a final volume of 50 mL of extract. The samples were filtered and stored under refrigeration.

Analysis of triterpene saponins

The HPLC analysis was performed using a chromatograph (Shimadzu, Sil 10 AF), with 20 μL injection with a UV detector (203 nm) and a LC-10AT pump, DGU-14A degasser and CTO-10AS oven at 35 °C and flow 1.0 mL min^{-1}. The ursolic acid was analyzed using a C-18 column, 4 μm 150 mm x 4.5 mm (Kromasil). The mobile phase was a mixture of acetonitrile: water (70:30 v/v). The solution was degassed by ultrasound and vacuum-filtered through a membrane (Millipore, PVDF). The samples were injected in triplicate and the peak areas compared with the ursolic acid standard (Sigma).

Analysis of methylxanthines and chlorogenic acid

The measurements were performed using chromatograph (Shimadzu Mod SLC-20A) equipped with a SIL-20AT injector, a LC-20 AT pump, DGU-20AS degasser and a CTO-20AS oven maintained at 30 °C. The C-18 column (Supelco LC-18 - 4.6 x 250 mm, 5 nm) was used, with injection of 20 μL sample and an interphase (Mod. Shimadzu LC solutions Release 1.22 SP1). The mobile phase was a gradient of (A): acidified water with 0.3% acetic acid (pH 3.0) and (B): methanol. Gradient: 15% to 20% B in 20 min, 20% to 85% B in 5 min and 85% B in 5 min. Flow 1.0 mL min^{-1}. Detection was monitored in a UV-photodiode array detector (UV-PDA; Shimadzu SPD-20A) at 325 nm for chlorogenic acid and 265 nm for caffeine and theobromine. Samples were injected in triplicate and the peak areas compared with the respective standards of chlorogenic acid, caffeine and theobromine (Sigma Chemical) (Cardozo Junior et al. 2007).

Calibration curve

The ursolic acid standard was dissolved in acetonitrile at concentrations of 8.75 to 175.0 μg mL (y = 9597.9 x + 846.88 r^2 = 0.9998). The caffeine standard was dissolved in the mobile phase and the scale was linear from 0.025 to 0.4 mg mL^{-1} (y = 5E+07x – 442141 r^2=0.9992) for theobromine from 0.05 to 0.4 mg mL^{-1} (y = 6E+07x – 78571 r^2=1) and chlorogenic acid from 0.025 to 0.4 mg mL^{-1} (y = 6E+07x – 362988 r^2=0.9995).

Evaluation of Antioxidant Activity

The reduction of the radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined as described by Schinella et al (2000), with adjustments. The methanolic extracts of the *I. paraguariensis* progenies were analyzed at dilutions of 2.0, 1.0, 0.5, 0.250, 0.125 and 0.0612 mg mL^{-1} of each sample with methanol PA and analyzed in a spectrophotometer after 20 minutes (absorbance 515 nm,). The potential of antioxidant activity was calculated in relation to the percentage of quercetin reduction (0.18 mg mL^{-1}) used as reference; the analysis was carried out in triplicate.

Data analysis

Data were submitted to analysis of variance, using the statistical model $Y_{ij} = \mu + G_i + B_j + \varepsilon_{ij}$ where Y_{ij} is the observation obtained for genotype i in plot j; μ is the overall mean, G_i is the effect of genotype i (random), B_j the effect of block j and ε_{ij} the random error. Heritability estimates in the broad sense were obtained from the mean square expectations of the analysis of variance:

$$h^2 = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_f^2} = \frac{(MSP - MSE)/r}{MSP/r}$$; where MSP and MSE are mean square of progeny and mean square of error, and r is the replication number.

A simple correlation analysis of the data was performed. Statistical analysis was performed using the Genes program (Cruz 2006).

RESULTS AND DISCUSSION

Eight progenies were selected for the evaluation of phytochemical compounds by clustering analysis (UPGMA). This method allows the analysis of genetic distance and groups progenies with the highest similarity degree together. The selection included a total of 51 progenies (Figure 1), based on data of

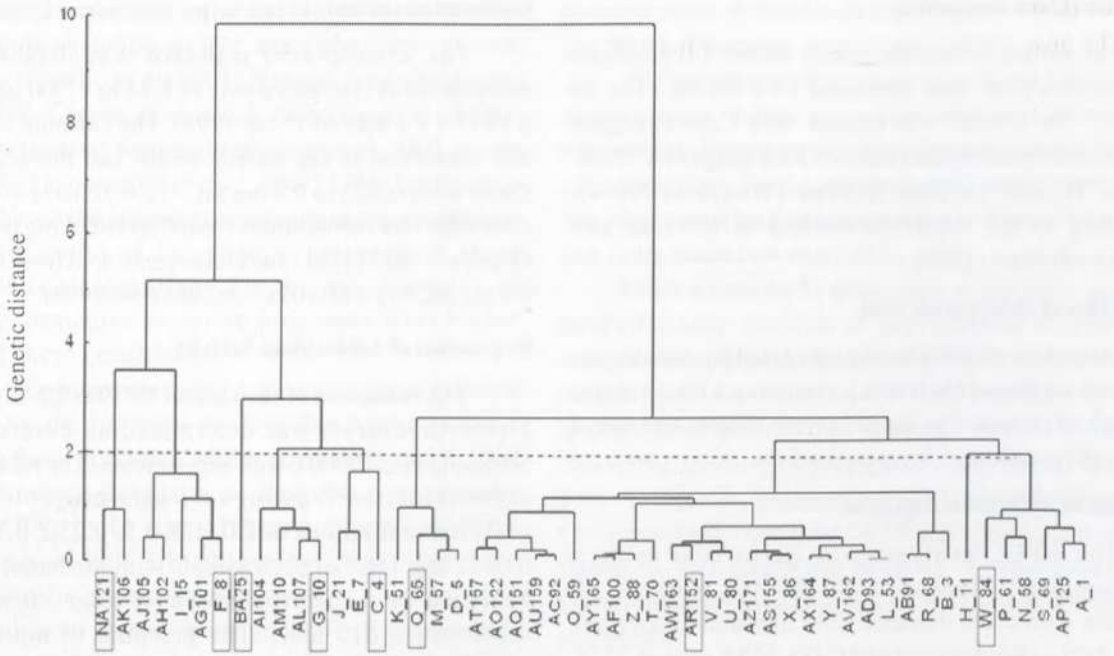

Figure 1. Clustering analysis by the UPGMA method, including 51 mate progenies, using data of Mahalanobis' generalized distance, based on the composition of methylxanthines and phenolic compounds. The horizontal dotted line indicates the cutting point for group formation. The selected progenies are marked with a rectangle

methylxanthine and phenolic compound levels.

The eight selected progenies were evaluated by HPLC, which allowed the simultaneous analysis of caffeine and theobromine at a wavelength of 265 nm and a retention time (RT) of 20 and 7.7 minutes, respectively, and chlorogenic acid at 325 nm with RT of 15.1 minutes. The triterpene saponins derived from ursolic acid were evaluated at 203 nm and a RT of 16 minutes.

The analysis of variance for the contents of ursolic acid, caffeine, theobromine and chlorogenic acid and antioxidant activity in all eight analyzed progenies (Table 1) showed significant variation in the evaluated compounds of the eight progenies, except for chlorogenic acid by the F test.

Several factors may influence the variability in the levels of the compounds tested, as part of the responses to environmental adaptation or genetic variation. The data of heritability (Table 1) show that the variability observed in the progenies evaluated here show a significant genetic component, as reported in the

Table 1. Summary of the analysis of variance of the heritabilty for the contents of triterpene saponines derived from ursolic acid, caffeine, theobromine, chlorogenic acid and antioxidant activity in eight mate (*Ilex paraguariensis* St. Hil.) progenies. Values expressed in %

	Ursolic acid	Caffeine	Theobromine	Chlorogenic acid	Antioxidant Activity
DF	14	14	14	14	14
MSB	0.000006	0.096769	0.24467	0.257996	200.931448
MSP	0.002294*	0.488321**	0.134776*	1.058429^{ns}	203.792746*
MSE	0.000571	0.106514	0.044657	0.9978	65.726043
h^2	75.09%	75.19%	66.87%	52.86%	67.75%
C.V.	52.49%	50.56%	49.98%	17.27%	20.48%

MSB: mean square of block; MSP: mean square of progeny; MSE: mean square of error
* and ** significant at 5% and 1% probability, respectively, by the F test
^{ns} Non-significant

literature (Cardozo Junior et al. 2007).

The quantification of triterpene saponins in mate is recent and knowledge is still scarce, but has attracted interest due to the biological activities. The reverse phase HPLC used here is considered the best technique and is widely used for saponin determination (Oleszek 2002). In this study, the variability in the levels of ursolic acid in the eight progenies had a strong genetic component, as indicated by the high heritability (Table 1).

The lowest content of ursolic acid was found in progeny AR152 (0.003%) and the highest in W84 (0.080%) (Table 2). Gnoatto et al (2005) reported a concentration of 352 μg mL^{-1} in the aqueous extract of mate leaves and a concentration of 704 μg mL^{-1} in the saponosidic fraction. In an analysis of green mate fruits, Pavei et al. (2007) detected 30.48 g% total saponins, while Vigo et al. (2004) found higher saponin contents (17.1 ± 0.2%) in *Pfaffia glomerata* in winter and spring, indicating environmental influence on the contents of this compound.

Environmental and genetic factors must be investigated to be able to respond to consumers who seek a product with milder flavor, but also to industrial demands, for which plants with higher contents of active principle are required. The results of this study are of highly relevant and can be a stimulus for further research on genetic improvement of triterpene saponin contents in mate.

The methylxanthine levels of mate are extremely variable within the species. The caffeine content of *I. paraguariensis* is higher than in other species; different harvest times influence the composition of this phytochemical (Coelho et al. 2001, Da Croce 2002, Schubert et al. 2006). The origin and genetic variability (Scherer et al. 2002, Costa et al. 2005, Cardozo Junior et al. 2007) are relevant in the breeding of plants with appropriate levels for consumption and industry. The caffeine levels observed here ranged from 0.23% (w / s) in progeny F8 to 1.38% in progeny Q65. These values are similar to those found in caffeine (from 0.25 to 1.66%) in samples collected in the half-sib trial in Ivaí-PR, in a joint analysis of progenies and provenances, which confirms the great genetic variability in the studied progenies. It is important to emphasize that in our study, caffeine levels in the progenies C4, F8, BA25 and NA121 were very low. Scherer et al. (2002) found a significant difference in caffeine and theobromine levels in mate progenies grown in Missiones, Argentina.

In the selection of mate progenies, the caffeine and theobromine levels are basic parameters in the search for the development of products with stimulating and tonic effects. But with the increasing demand for decaffeinated products, the selection of progenies with low caffeine levels may be an alternative to traditional mate consumers that have problems with heart diseases, insomnia or anxiety, aggravated by the action of the stimulant caffeine.

The theobromine concentrations measured in this study, ranging from 0.176% (Q65) to 0.83% (NA121) were comparable to the values (0.106 to 0.807%) for the same progenies, confirming the existence of variability of this compound in mate. It is worth highlighting the importance of selecting a progeny with extreme caffeine and theobromine values in an improvement program with a view to breeding alternative products with a pre-defined composition of the chemical traits of interest.

Table 2. Mean contents of triterpene saponines derived from ursolic acid, caffeine, theobromine, chlorogenic acid and antioxidant activity analyzed in a 0.5 mg mL^{-1} sample solution of eight mate (*Ilex paraguariensis* St. Hil.) progenies. Values expressed in %

Mate progeny	Ursolic acid	Caffeine	Theobromine	Chlorogenic acid	% Inhibition
C4	0.043	0.440	0.361	1.467	34.836
F8	0.031	0.230	0.515	1.651	50.901
GI0	0.068	0.607	0.468	1.365	31.251
BA25	0.041	0.226	0.504	2.031	51.406
Q65	0.077	1.377	0.176	1.501	44.459
W84	0.080	1.027	0.177	1.344	31.276
NA121	0.022	0.447	0.831	1.603	36.877
AR152	0.003	0.810	0.350	1.401	35.633

The concentration of chlorogenic acid observed here ranged from 1.34% to 2.03%, with significant differences between the progenies. These values are close to those reported by Filip et al. (2001), of 2.8 ± 3% in *I. paraguariensis*. Chlorogenic acid is found at low concentrations in mate (Clifford and Ramirez-Martinez 1990, Filip et al. 2001) with a predominance of the di-caffeoylquinic derivatives (3,4-DCQ, 3,5-DCQ and 4,5 — DCQ). The low coefficient of variation (17.27%) (Table 1) suggests that this compound is less related to environmental conditions.

The progenies BA25, F8 and NA121 had the highest chlorogenic acid levels, which will require further studies with these progenies to obtain clones with high antioxidant levels, due to the presence of this compound. In the correlation analysis (Table 3) the chlorogenic acid content and antioxidant activity were positively correlated, although not significantly, by the t test.

Bioactive compounds in mate, such as phenolic compounds and the derivatives caffeoyl and flavonoids, have an antioxidant activity *in vivo* and *in vitro* (Gugliucci 1996, Bastos et al. 2006a, Bastos et al. 2006b) an inhibitory capacity of lipid peroxidation (Schinella et al. 2000), they influence LDL (low density lipoprotein) oxidation *in vitro* and *in vivo*, and they act as protective agents against cardiovascular and gastrointestinal diseases, skin cancer and fungus proliferation (Soares 2002, Schinella et al. 2005). This reinforces the importance of the selection of mate progenies with a high content of antioxidant compounds. Products with greater health benefits for consumers could be obtained from selected progênies planted in a clonal orchard.

Mate breeding programs have focused on the production of leaf biomass (Costa et al. 2005). But genetic parameters (Scherer et al. 2002, Costa et al. 2005, Cardozo Junior et al. 2007) have recently been estimated to determine the phytochemical potential of mate to select progenies with compound levels that would satisfy the commercial and industrial sector.

The heritability estimates for the caffeine (75.19%), theobromine (66.87%), chlorogenic acid (52.86%) and saponin contents (75.09%) in the tested *I. paraguariensis* progenies confirmed that the genetic factor is important in the variability of phytochemical compounds in these progenies. High heritability estimates for caffeine, tannin and polyphenol levels were also reported elsewhere (Scherer et al. 2002, Sturion et al. 2004). These results indicate that it is possible to modify the caffeine, theobromine and saponin contents by selection strategies aiming at higher levels of the compound of interest.

These heritability values indicate possible gains in inter-and intra-progenies selection in mate breeding programs. The variability among progenies, along with the high trait heritability, indicate that it is possible to obtain progenies with a more appropriate composition of these phytochemicals, which will result in a product with more health benefits for consumers. Moreover, these progenies can be used as raw material for the purification of these compounds and in the prevention and treatment of diseases triggered by free radicals.

Table 3 shows the correlation coefficients among the characteristics evaluated. The levels of caffeine are negatively and significantly correlated with chlorogenic acid (-0.43) and theobromine (-0.58). This negative correlation can be explained since caffeine and

Table 3. Correlation between the triterpene saponines derived from ursolic acid, caffeine, theobromine, chlorogenic acid and antioxidant activity in eight mate (*Ilex paraguariensis* St. Hil.) progenies

	Saponins	Caffeine	Theobromine	Chlorogenic acid	% Antioxidant activity
Saponins	1.00				
Caffeine	0.38	1.00			
Theobromine	-0.55*	-0.58*	1.00		
Chlorogenic acid	-0.14	-0.43*	0.43*	1.00	
% Antioxidant activity	0.00	0.23	-0.01	0.36	1.00

* Significant at 5% probability by the t test

theobromine share the same biosynthetic pathway, where caffeine is synthesized from xanthosine –> 7-methylxanthosine –> 7-methylxanthine –> theobromine and the methyl donor is S-adenosylmethionine (SAM). A negative correlation between these traits was also stated by Cardozo Junior et al. (2007) and Gnoatto et al. (2007). Theobromine is also negatively correlated with saponins (-0.55), and positively with chlorogenic acid (0.43). This indicates that a simultaneous increase in the levels of theobromine with caffeine and saponins in the breeding program is likely to be difficult. It will also be complicated to increase the levels of caffeine and chlorogenic acid simultaneously, whereas a simultaneous accumulation of theobromine and chlorogenic acid should be possible.

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Variação genética nos teores de compostos fitoquímicos em progênies de *Ilex paraguariensis* St. Hil.

RESUMO - *A erva-mate (*Ilex paraguariensis *St. Hil.) possui compostos fitoquímicos capazes de prevenir uma série de problemas de saúde. O conhecimento da contribuição genética na variabilidade destes compostos pode auxiliar os programas de melhoramento a obter progênies de erva-mate com níveis melhorados destes compostos. Neste trabalho foram avaliadas a composição de saponinas triterpênicas, metilxantinas, ácido clorogênico e a atividade antioxidante de 8 progênies de erva-mate. Os resultados evidenciaram uma diferença significativa entre as progênies nos teores de saponinas triterpênicas (0,003-0,080%) cafeína (0,226-1,377%), teobromina (0,176-0,831%), ácido clorogênico (1,344-2,031%) e atividade antioxidante (31,251-51,406%). Foi encontrada uma correlação positiva entre o teor de ácido clorogênico e a atividade antioxidante, e negativa entre teobromina com cafeína e saponinas, e de cafeína com ácido clorogênico. O coeficiente de herdabilidade para saponinas (75,09%), cafeína (75,19%) e teobromina (66,87%), ácido clorogênico (52,86%) e na atividade antioxidante (67,75%) indicam a possibilidade de ganhos genéticos por seleção para estas características.*

Palavras-chave: *Aquifoliaceae,* saponinas triterpênicas, metilxantinas, ácido clorogênico, atividade antioxidante.

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