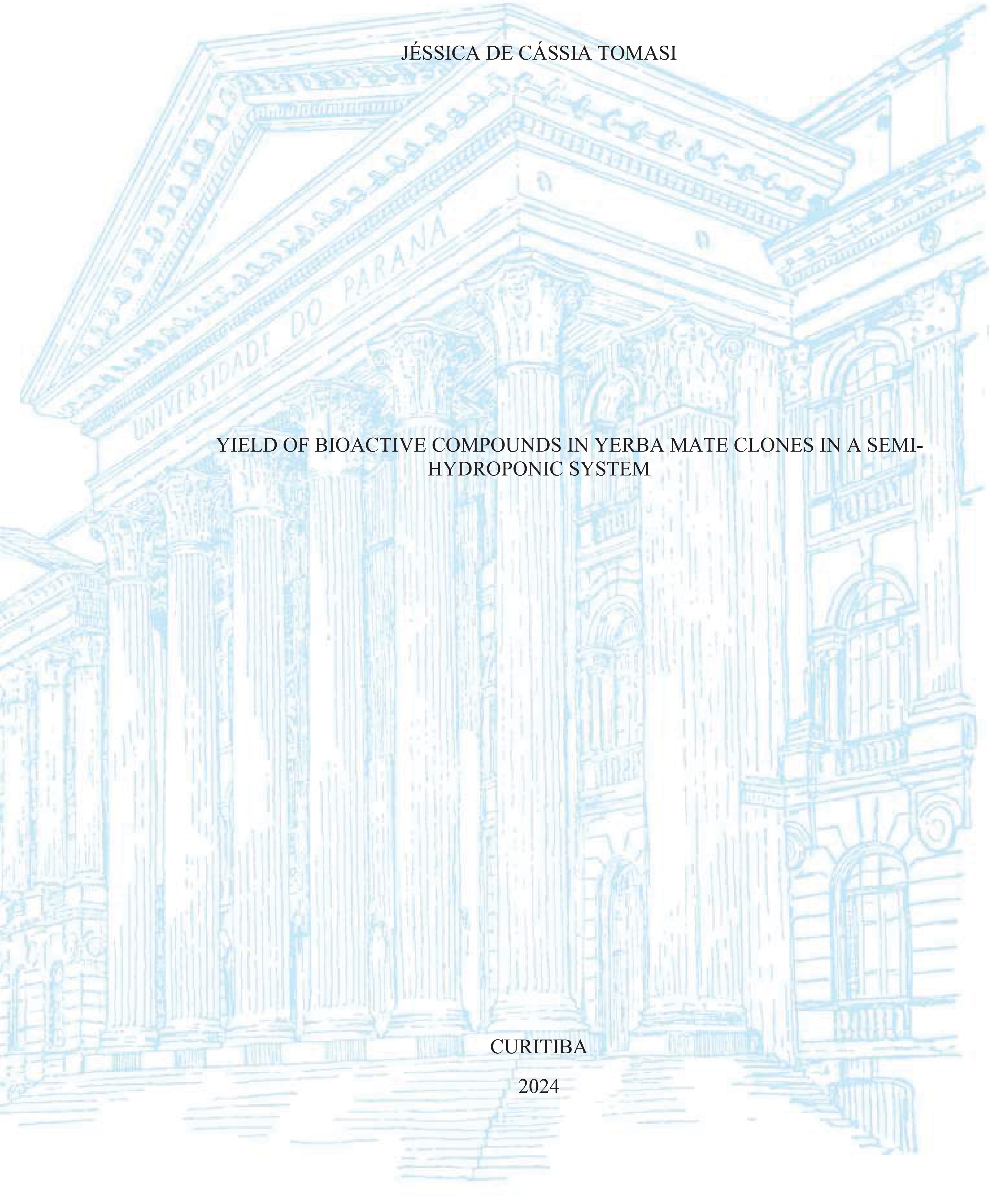
UNIVERSIDADE FEDERAL DO PARANÁ

JÉSSICA DE CÁSSIA TOMASI



YIELD OF BIOACTIVE COMPOUNDS IN YERBA MATE CLONES IN A SEMI-HYDROPONIC SYSTEM

CURITIBA

2024

JÉSSICA DE CÁSSIA TOMASI

YIELD OF BIOACTIVE COMPOUNDS IN YERBA MATE CLONES IN A SEMI-HYDROPONIC SYSTEM

Tese apresentada ao curso de Pós-Graduação em Engenharia Florestal, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Engenharia Florestal.

Orientador: Prof. Dr. Ivar Wendling

Coorientadora: Drª. Cristiane Vieira Helm

Coorientadora: Drª. Manoela Mendes Duarte

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA FLORESTAL da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JÉSSICA DE CÁSSIA TOMASI** intitulada: **"YIELD OF BIOACTIVE COMPOUNDS IN YERBA MATE CLONES IN A SEMI-HYDROPONIC SYSTEM"**, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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"Esforçai-vos e tende bom ânimo; não temais, nem vos espanteis, porque o Senhor, vosso Deus, é convosco, por onde quer que andardes."

Josué 1:9

RESUMO

O conhecimento dos fatores que interferem na composição química da erva-mate (*Ilex paraguariensis*) permite a obtenção de matéria-prima de qualidade, com perfis fitoquímicos específicos para mercados distintos. Para tanto, o trabalho foi dividido em dois capítulos, sendo um deles uma revisão sistemática e o segundo um estudo sobre o rendimento de compostos bioativos em erva-mate em sistema semi-hidropônico. No primeiro capítulo, fornecemos uma visão geral de como os fatores agronômicos e de processamento podem influenciar no teor dos compostos bioativos presentes nas folhas de erva-mate. Para tanto, utilizamos a metodologia PRISMA para selecionar artigos publicados em periódicos indexados que inter-relacionem fatores relativos ao manejo do cultivo e fatores de processamento às características fitoquímicas de *I. paraguariensis*. Identificamos que poucos estudos se concentram nessa inter-relação. Apesar disso, há uma tendência no desenvolvimento de novas pesquisas sobre o tema, tendo em vista a elevada versatilidade da espécie e seu espaço em mercados consumidores diversos. Os resultados desse estudo podem auxiliar no direcionamento de novos caminhos para pesquisas, contribuindo também com o setor produtivo da erva-mate. Já no segundo capítulo, analisamos o teor e o rendimento de compostos bioativos em dois clones de erva-mate submetidos a doses crescentes de nitrogênio durante dois anos em sistema de cultivo semi-hidropônico. As folhas foram classificadas como jovens ou maduras e moídas após secagem em micro-ondas. Os extratos aquosos foram analisados em Cromatógrafo Líquido Ultra-Rápido (UFLC). O rendimento foi calculado multiplicando-se o conteúdo do composto pela massa seca das folhas. As folhas jovens apresentaram níveis mais elevados de praticamente todos os compostos do que as folhas maduras. O clone EC40 apresentou maiores teores de cafeína, 4-CQA (ácido 4-cafeoilquínico) e 5-CQA (ácido 5-cafeoilquínico), bem como, maior rendimento de todos os compostos, exceto teobromina, quando comparado ao EC22. O aumento das doses de nitrogênio aumentou os níveis de metilxantinas; entretanto, o rendimento dos compostos diminuiu com níveis mais elevados de N devido à redução da massa foliar. Com a dose de máxima de produtividade, 206 mg L^{-1} de N, a produtividade do composto atingiu até 21 g m^{-2} ano^{-1} de cafeína e 126 g m^{-2} ano^{-1} de CQAs no clone EC40, e 14 g m^{-2} ano^{-1} de teobromina em EC22. Estes resultados demonstram que o sistema de cultivo proposto é viável para produção de erva-mate para extração industrial de compostos bioativos. Matérias-primas com características fitoquímicas específicas podem ser obtidas através da seleção de clones e tipos de folhas.

Palavras-chave: Compostos fenólicos. Cultivo. Metabólitos secundários. Metilxantinas. Secagem. Nutrição de plantas.

ABSTRACT

Understanding the factors that affect the chemical composition of yerba mate (*Ilex paraguariensis*) enables the acquisition of high-quality raw material with specific phytochemical profiles for different markets. So, the work was divided into two chapters, one being a systematic review and the second a study on the yield of bioactive compounds in a semi-hydroponic system. In the first chapter, we provide an overview of how agronomic and processing factors can influence on the content of bioactive compounds present in yerba mate leaves. To achieve this, we employed the PRISMA methodology to select articles published in indexed journals that interrelated cultivation management and processing factors to the phytochemical characteristics of *I. paraguariensis*. We identified that few studies focus on this interrelation. Nevertheless, there is an increasing trend in the development of new research on the subject, considering the high versatility of the species and its presence in various consumer markets. The results of this study can assist in directing new paths for research, also contributing to the yerba mate production sector. In the second chapter, we analyzed the content and yield of bioactive compounds in two clones of yerba mate submitted to increasing doses of nitrogen for two years in a semi-hydroponic cultivation system. The leaves were classified as young or mature and ground after drying in a microwave. The aqueous extracts were analyzed using an Ultra-Fast Liquid Chromatograph (UFLC). The yield was calculated by multiplying compound contents by the leaf dry mass. Young leaves presented higher levels of virtually all compounds than mature leaves. Clone EC40 showed higher levels of caffeine, 4-CQA (4-caffeoylquinic acid), and 5-CQA (5-caffeoylquinic acid), and this genotype showed a higher yield of all compounds, except for theobromine, when compared to EC22. Increasing nitrogen doses increased methylxanthine levels; however, the yield of compounds decreased with higher N levels due to reduced leaf mass. With the maximum productivity dose, 206 mg L^{-1} of N, the compound productivity reached up to 21 g m^{-2} $year^{-1}$ of caffeine and 126 g m^{-2} $year^{-1}$ of CQAs in clone EC40, and 14 g m^{-2} $year^{-1}$ of theobromine on EC22. These results demonstrate that the proposed cultivation system is viable for producing yerba mate for the industrial extraction of bioactive compounds. Raw material with specific phytochemical characteristics can be obtained by clones and leaf types selecting.

Keywords: Phenolic compounds. Cultivation. Secondary metabolites. Methylxanthines. Drying. Plant Nutrition.

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GENERAL INTRODUCTION

Originating from the subtropical region of South America, specifically from Brazil, Paraguay and Argentina, yerba mate, known scientifically as *Ilex paraguariensis*, stands out as an element of significant importance in the social, cultural and economic contexts of the producing regions. Over the centuries, it has played a fundamental role, shaping traditions and influencing various industrial sectors. Its versatility, which goes beyond simple traditional beverages (*chimarrão* and *tererê*), covers several sectors, such as the composition of foods and beverages, as well as cosmetics and pharmaceuticals.

Traditionally, the yerba mate production chain involves field cultivation, harvesting, processing and distribution of the raw material to different sectors of the national economy (food, cosmetics and others); Furthermore, the international market has increasingly recognized the value of this plant, boosting exports and consolidating it as a product of global relevance.

The interest garnered towards this species and its potential arise from the bioactive compounds naturally present in its leaves, which offer several benefits to human health. The benefits of compounds such as methylxanthines (caffeine and theobromine), phenolic compounds (phenolic acids and flavonoids) and saponins in yerba mate are widely known and consolidated in the literature. However, what is still lacking is further information and clarity over the influence of agronomic and processing factors on these bioactive compounds and how we can use this knowledge to guide the production of chemically potential raw materials for different industrial sectors.

In terms of agronomic factors, the concentration and accumulation of secondary metabolites are influenced by genetic factors (genetic regulation), ontogenic factors (developmental stages), morphogenetic factors (tissues which performed specialized functions) and environmental factors (abiotic and biotic). In case of the environmental many abiotic factors are involved in fluctuation of secondary metabolites with water, light, temperature, soil and chemicals (minerals/fertilizers) and can redirect the metabolism to consequently regulate the production of active constituents. Because plants have a complex and variable chemical composition, the type and content of secondary metabolites as well as biological effects were often determined according to the change of environment.

In terms of processing factors, the content of bioactive compounds, as well as the aroma and taste, are influenced mainly by post-harvest (drying) that directly interferes with the final quality of raw material that will be used in various industrial sectors.

Therefore, this research aimed to make a literature review, seeking to compile all the information generated in recent years involving yerba mate and the effects of agronomic and processing factors on bioactive compounds. The goal was not only to clarify this topic, but also to guide future research. Furthermore, a study was conducted on an innovative cultivation system (semi-hydroponic) with selected genetic materials to evaluate the impact of nitrogen on the content of bioactive compounds, seeking to add a new cultivation method to the crop and contribute significantly to Brazilian agriculture.

CHAPTER I - INFLUENCE OF AGRONOMIC AND PROCESSING FACTORS ON BIOACTIVE COMPOUNDS OF YERBA MATE: A SYSTEMATIC REVIEW

ABSTRACT
Understanding the factors that affect the chemical composition of yerba mate (*Ilex paraguariensis*) enables the acquisition of high-quality raw material with specific phytochemical profiles. In this review, we provide an overview of how agronomic and processing factors can influence on the content of bioactive compounds present in yerba mate leaves. To achieve this, we employed the PRISMA methodology to select articles published in indexed journals that interrelated cultivation management and processing factors to the phytochemical characteristics of I. paraguariensis. We identified that few studies focus on this interrelation. Nevertheless, there is an increasing trend in the development of new research on the subject, considering the high versatility of the species and its presence in various consumer markets. The results indicate divergence among the studies regarding the effect of various factors (light, seasonality, leaf age, sexual dimorphism). However, the influence of genetic factors on the content of bioactive compounds is clearly evident. For processing, the main findings highlight that scalding helps maintain bioactive compounds; however, oxidation and toasting processes primarily result in a reduction of phenolic compounds. The results of this study can assist in directing new paths for research, also contributing to the yerba mate production sector.

Keywords: Phenolic Compounds, Cultivation, *Ilex paraguariensis*, Secondary Metabolites, Methylxanthines, Drying.

1 INTRODUCTION

Yerba mate (*Ilex paraguariensis* A.St.-Hil.) has gained prominence in the global scientific scenario, with numerous studies emphasizing its consumption and benefits to human health, particularly its antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, anti-obesity, and cardioprotective properties (GÓMEZ-JUARISTI et al., 2018; VALDUGA et al., 2019), as well as hepatoprotective effects (TAMURA et al., 2013), neuroprotective effects (BRANCO et al., 2013), and hypolipidemic effects (BRAVO et al., 2014). The versatility and numerous benefits of yerba mate are attributed to the wide variety of bioactive compounds present in its leaves, such as methylxanthines, saponins, and phenolic compounds like flavonoids and caffeoylquinic acids (VALDUGA et al., 2019). These and other compounds have attracted the interest of national and international markets, leading to the use of yerba mate in the manufacturing of various nutraceutical, cosmetic, and pharmaceutical products (HECK & DE MEJIA, 2007; SOUZA et al., 2015; CARDOZO JUNIOR & MORAND, 2016; BECKER et al., 2019; CROGE et al., 2021).

In yerba mate, the methylxanthines found in higher concentrations are caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) (YIN; KATAHIRA; ASHIHARA, 2015). Caffeine offers various benefits for human consumption, acting as a central nervous system stimulant, cardiovascular function enhancer, and diuretic; whereas theobromine has a similar effect to caffeine but with less intense activity, in addition to being broncho- and vasodilatory, diuretic, and muscle relaxant (Smit, 2011; Ma et al., 2018). The species is also an important source of phenolic compounds (GEBARA et al., 2017), especially chlorogenic acids, mono- and dicaffeoylquinic acids (MATEOS et al., 2018).

The content of bioactive compounds can be directly influenced by agronomic factors, especially those related to cultivation and crop management (Dutra et al., 2010; Berté et al., 2014). Environmental conditions such as seasonality, temperature, radiation, water availability, and nutrient availability influence the biosynthesis and storage of secondary metabolites (Dutra et al., 2010), as the expression of involved genes is altered by different biotic or abiotic factors (Li et al., 2020). Genetic factors also play a significant role in compound synthesis, being crucial in selecting genotypes with specific chemical characteristics (De Morais et al., 2009; Barbosa et al., 2015; Blum-Silva et al., 2015; Cardozo Junior & Morand, 2016; González Arbeláez et al., 2016).

Photoperiod and light intensity are essential components for photosynthesis and plant growth (LI et al., 2018; ZHANG et al., 2015), affecting the levels of methylxanthines, flavonoids, and phenolic acids (Nakamura et al., 2009; Junior et al., 2010; Li et al., 2020), among other compounds. Leaf age (BLUM-SILVA et al., 2015) and harvest time (Schubert et al., 2006) are also described as factors affecting the synthesis of bioactive compounds in yerba mate. Fertilization, especially nitrogen-based, also interferes with the synthesis of bioactive compounds in the species (Rossa et al., 2017; Westphalen et al., 2020; Tomasi, 2020).

Literature demonstrates that industrial processing of leaves, such as blanching, drying, grinding, and toasting, influences bioactive compounds (DARTORA et al., 2011; DUARTE et al., 2020; RIACHI et al., 2018; VALERGA; RETA; LANARI, 2012). During these processes, changes in the content of bioactive compounds can occur, influencing the characteristics of the beverage, particularly flavor. These alterations impact the consumption and acceptance of yerba mate beverages and understanding them is essential for obtaining products with known phytochemical profiles, enabling targeting specific markets (DUARTE et al., 2020; TOMASI et al., 2021b).

Thus, our systematic review aimed to clarify the phytochemical response of yerba mate in different production systems and crop management, identifying agronomic factors that influence the content of bioactive compounds in the species. With this information, we can manage systems more efficiently, seeking high-quality raw materials with desired phytochemical profiles. Additionally, we explored scientific literature for factors related to the processing of raw materials that may influence the content of bioactive compounds in the final product, aiming to elucidate the impact of these processes on the chemical characteristics and quality of the product.

2 MATERIAL AND METHODS

The literature review was conducted following the PRISMA methodology (Moher et al., 2009; Fig. 2). We used two electronic indexing databases – Web of Science and Scopus – to identify available literature and cover a wide range of high-quality journals. Our search was limited to research articles published in scientific journals in English, Portuguese, and Spanish. Review articles, conference papers, technical reports, and technical communications were excluded. There was no restriction on the publication period (year of publication) for the initial search date and the final search date was August 2023.

2.1 CRITERIA FOR SELECTING THE INFLUENCE OF AGRONOMIC FACTORS ON THE PRODUCTION OF BIOACTIVE COMPOUNDS IN YERBA MATE

We sought published studies that linked the content of bioactive compounds in *I. paraguariensis* with agronomic production factors of the raw material: seasonality, luminosity, cultivation system, leaf development stage (type of leaf), planting density, fertilization, irrigation, soil management/preparation, and genetic material. Studies evaluating the effect of different cultivation systems, with the primary focus on light incidence, were included under the luminosity factor.

Title, abstract, and keywords were searched using the set of words: "*Ilex paraguariensis*," "erva mate," "yerba mate," "bioactive compounds," "methylxanthines," "caffeine," "theobromine," "caffeoylquinic acids," "secondary metabolites," "saponins," and "flavonoids," with the application of Boolean operators, for example, OR and AND. Our database search returned 236 articles in the Web of Science and 375 articles in Scopus. A total of 441 articles remained after removing duplicates.

During the screening process, after reading titles and abstracts, articles were analyzed and excluded if they: (a) focused on the processing phase of plant material or the storage, industrialization, and commercialization of the product; (b) related agronomic factors to morphophysiological aspects of plants; (c) focused on other parts and organs of plants, not leaves; (d) analyzed in the area of human health; and (e) analyzed in the area of food; and (f) only examined primary metabolites. Articles were included if they: (a) included analyses of bioactive compounds present in yerba mate leaves, combined with one or more agronomic factors. After applying these criteria, 28 articles remained potentially useful for full-text reading.

The remaining articles were evaluated as full texts for the clarity of the information provided. Articles were excluded if they: (a) assessed the content of bioactive compounds in tissue culture; and (b) assessed the effect of agronomic factors on the mineral composition of leaves. Following this second set of eligibility criteria, 26 articles were included in this review.

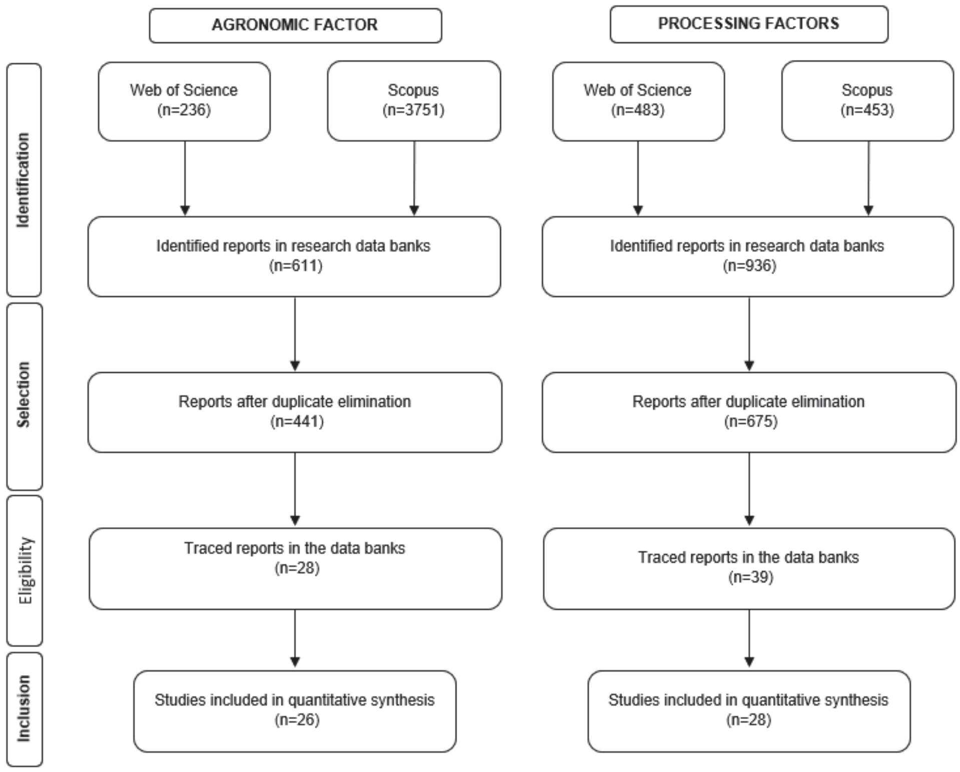

Figure 1. Workflow description of the PRISMA research methodology, adapted from Moher et al. (2009).

2.2 CRITERIA FOR SELECTING THE INFLUENCE OF RAW MATERIAL PROCESSING STEPS ON THE PRODUCTION OF BIOACTIVE COMPOUNDS IN YERBA MATE

We searched existing literature for published studies that correlated the content of bioactive compounds in *I. paraguariensis* with different processing methods, such as drying, toasting, blanching, drying temperatures, grinding, freeze-drying, oxidation, and aging/storage. Title, abstract, and keywords were searched using the words "*Ilex paraguariensis*," "erva mate," "yerba mate," "bioactive compounds," "methylxanthines," "caffeine," "caffeoylquinic acids," "secondary metabolites," "phenolic compounds," "drying," "toast," and "processing," employing Boolean operators, for example, OR and AND. Our database search returned 483 articles in Web of Science and 453 articles in Scopus. A total of 675 articles remained after removing duplicates.

During the screening process, after reading titles and abstracts, articles were analyzed and excluded if they: (a) focused on the product storage phase; (b) assessed infusions of commercial products; (c) evaluated compound extraction methods; (d) analyzed in the area of human health; (e) focused on the effect of processing on enzymatic activation; and (f) analyzed the effect of processing on nutritional content. After applying these criteria, we obtained 39 articles in the database potentially useful for full-text reading.

The remaining articles were evaluated as full texts for the clarity of the information provided. Articles were excluded if they: (a) dealt with the industrial processing of extracts for encapsulation and not the processing of raw yerba mate material; (b) evaluated the effect of treatment with ionizing radiation on already processed materials; (c) focused on the analysis of drying methods on extraction yield rather than chemical composition; (d) were reviews that were not excluded in the database filters; and (e) compared a processing method with commercially packaged products without specifying the processing used for these products. Following this second set of eligibility criteria, we included 28 articles for this review.

2.3 DATA ANALYSIS

Descriptive statistics were used to analyze general trends regarding the frequency of publications, the number of studies published by country, as well as by Brazilian states, and the number of studies per agronomic factor. All selected articles were analyzed to gather information. We extracted all relevant information related to these variables and, for each article, we classified the results as neutral (*), positive (+), or negative (-) in response to agronomic or processing factors. From this classification, we generated tables presenting the factors and the response regarding secondary metabolites.

3. RESULTS AND DISCUSSION

3.1 AGRONOMIC FACTORS AND BIOACTIVE COMPOUNDS

Few studies interrelate agronomic factors with the biosynthesis of bioactive compounds in *I. paraguariensis*; only 26 publications met the pre-established criteria in this review. Brazil has the highest number of publications (21), followed by Argentina (4) and Japan (1). All Brazilian publications were conducted in the Southern region of the country, with a notable presence in the state of Paraná, accounting for 11 studies. We observed that research from this perspective began in the 2000s, with fluctuations in the number of publications between 2002 and 2023 (Table 1). In the last eight years, eleven publications have been conducted, suggesting an increasing trend in the number of studies focusing on this theme, driven by the prominence that yerba mate has been gaining in national and international markets. These studies can assist the productive sector in producing raw materials with specific phytochemical profiles depending on their final destination.

Table 1. Summary of published studies that related agronomic factors to the content of bioactive compounds in *Ilex paraguariensis.*

BIOACTIVE COMPOUND	AGRONOMIC FACTOR	RESULTS	REFERENCE	COUNTRY	STATE
ACG	Seasonality	ACG content at the beginning of the harvest (April/May) was substantially higher than at the end of the harvest (September).	(BUTIUK et al., 2016)	Argentina	Misiones
MXT	Seasonality	Decrease in the content of methylxanthines in two populations of *I. paraguariensis* during the late autumn and throughout winter.	(SCHUBERT et al., 2006)	Brazil	Rio Grande do Sul
MXT and CF	Leaf age	Reduction in the total methylxanthine, caffeine, and phenolic compound content with increasing leaf age.	(BLUM-SILVA et al., 2015)	Brazil	Santa Catarina
C	Leaf age	No difference in caffeine content between young and mature leaves.	(YIN; KATAHIRA; ASHIHARA, 2015)	Japan	Kagoshi ma
MXT and CF	Leaf age e Light intensity	Mature leaves have a higher amount of theobromine. Monoculture with 100% solar radiation shows higher levels of caffeine and phenolic compounds.	(STRASSMANN et al., 2008)	Brazil	Santa Catarina
C and T	Leaf age and Light intensity	Increased light intensity and leaf age led to a reduction in caffeine and theobromine levels.	(ESMELINDRO et al., 2004)	Brazil	Rio Grande do Sul
MXT	Light intensity	Methylxanthine content increased in the treatment with low light intensity.	(COELHO et al., 2007)	Brazil	Rio Grande do Sul

C, T, ACG and CF	Light intensity	Full sun cultivation resulted in products with higher levels of caffeine, theobromine, chlorogenic acid, and phenolic compounds compared to shaded cultivation.	(HECK; SCHMALKO; DE MEJIA, 2008)	Argentine	Misiones
C and T	Cultivation systems (monoculture and forest)	Sun-cultivated leaves (monoculture) had a higher content of bioactive compounds compared to those cultivated in the shade (forest).	(DARTORA et al., 2011)	Brazil	Rio Grande do Sul
CF and Fl	Soil cover, Seasonality and light intensity	The accumulation of phenolic compounds, flavonoids, and antioxidant capacity was higher in 35% shaded treatments harvested in autumn.	(Ferrera et al., 2016)	Brazil	Rio Grande do Sul
MXT and CF	Light intensity and fertilization	Apparent light intensity of 30% and conventional fertilizers established the best indicator for obtaining methylxanthines.	(Rossa et al., 2017)	Brazil	Paraná
C, T and ACG	Light intensity	Caffeine and chlorogenic acid biosynthesis is favored in environments with more than 20% shading.	(BORGES et al., 2019)	Brazil	Santa Catarina
MXT	Light intensity	Increased caffeine content in plants subjected to 70% shading.	(TORMENA et al., 2020)	Brazil	Santa Catarina
CF and Fl	Light intensity	"Full sun" cultivation showed a higher concentration of phenolic acids and a lower concentration of flavonoids and saponins.	(LORINI et al., 2021)	Brazil	Santa Catarina
C	Cultivation systems (native, cultivated 5 years, cultivated 15 years)	No clear response of cultivation type on caffeine content.	(PAGLIOSA et al., 2009)	Brazil	Santa Catarina
MXT	Sexual dimorphism	Higher levels of MTX were found in leaves of male plants compared to female leaves, regardless of the phenophase and light microenvironment.	(PAULI et al., 2019)	Brazil	Paraná
C, T, ACG, and AC	Sexual dimorphism	No difference between genders in relation to bioactive compounds.	(RAKOCEVIC et al., 2023)	Brazil	Paraná
CA, CF, MXT, and AC	Genetic factor/morphotypes	Caffeine content differed only between dark green (higher) and "furry" (lower) morphotypes.	(DUARTE et al., 2022)	Brazil	Paraná
MXT and CF	Genetic factor	Significant differences were found in the content of caffeine, theobromine, rutin, and chlorogenic acid, as well	(VALDUGA et al., 2016)	Brazil	Rio Grande do Sul

		as antioxidant activity in selected progenies.			
MXT and AC	Genetic factor	High genetic influence on the levels of the evaluated compounds.	(FRIEDRICH et al., 2017)	Brazil	Rio Grande do Sul and Paraná
MXT and ACG	Genetic factor	Significant differences in the levels of all evaluated compounds among the progenies.	(NAKAMURA et al., 2009)	Brazil	Paraná
MXT, ACG and AC	Genetic factor	Caffeine and theobromine contents were significantly different depending on the place of origin, with high individual heritability. Two different progeny groups were determined for chlorogenic acid and caffeic acid.	(CARDOZO et al., 2010)	Brazil	Paraná
MXT and CF	Genetic factor	Significant differences in the content of total methylxanthines, caffeine, and theobromine in progenies, according to the place of origin.	(CARDOZO et al., 2007)	Brazil	Rio Grande do Sul and Paraná
C and T	Genetic factor	High variation in the levels of caffeine and theobromine in selected trees.	(MARX et al., 2003)	Argentine	Misiones
C and T	Genetic factor	High variation in the caffeine and theobromine content in selected progenies.	(SCHERER et al., 2002)	Argentine	Misiones
CF and MXT	Genetic factor	High variation in the phytochemical content of the evaluated genotypes.	(VIEIRA et al., 2021)	Brazil	Paraná

MXT: total methylxanthines; C: caffeine; T: theobromine; ACG: chlorogenic acid; CF: phenolic compounds.

Physiologically, secondary metabolites are produced by plants with the primary function of protection, allelopathic action, and as attractants for pollinating animals (GUERRIERI; DONG; BOUWMEESTER, 2019; ISAH, 2019; VERMA; SHUKLA, 2015). Thus, it can be stated that the production of many of these compounds is highly influenced by environmental conditions to which the plants are subjected, explaining the significant variation in content of each compound in different studies, as presented in Table 2. Evaluating the selected studies, the effect of agronomic factor luminosity was the most studied, with 10 publications (Table 3). The most commonly used cultivation systems for yerba mate are monoculture, usually in full sun with higher light incidence, and extractive exploitation (in the understory of the forest) and agroforestry system, where plants develop associated with other forest components and are shaded by the canopy of these trees (Santin et al., 2017a; Santin et al., 2017b; Santin et al., 2019; Westphalen et al., 2020). The most analyzed compound in these luminosity studies was caffeine, which is the most abundant methylxanthine in yerba mate, followed by theobromine and theophylline (VALDUGA et al., 2019). Many authors observed higher caffeine levels in plants subjected to shading greater than 70%, whether natural (forest) or artificial. Coelho et al. (2007) stated that caffeine levels were higher in plants in the understory of native forest, with natural shading exceeding 95%. Riachi et al. (2018) concluded that yerba mate leaves cultivated in natural shade (~90% shading) presented significantly higher concentrations of caffeine than those in full sun. Esmelindro et al. (2004) found caffeine levels around 3 times higher in plants subjected to 75% shading. Tormena et al. (2020) observed an increase in caffeine content in plants subjected to 70% shading, and Rossa et al. (2017) in plants subjected to an apparent luminosity of 30% (shading of 70%).

Table 2. Maximum and minimum concentrations of each compound found in the studies, expressed in mg g^{-1} of dry weight, and phenolic compounds expressed in (mgEAG g^{-1}).

Reference	Bioactive compounds											
	MxT[1]		C[1]		T[1]		ACG[1]		AC[1]		CF[2]	
	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min
(SCHUBERT et al., 2006)	10.37	1.77										
(BUTIUK et al., 2016)							80.80	22.70				
(FERRERA et al., 2016)												
(YIN; KATAHIRA; ASHIHARA, 2015)			15.00	23.00								
(BLUM-SILVA et al., 2015)[1]	137.05	6.20	133.98	5.68	30.88	0.26					437.81	75.27
(ESMELINDRO et al., 2004)[1]			350.94	42.36	4.14	0.11						
(COELHO et al., 2007)			21.46	0.20	8.45	0.47						
(HECK; SCHMALKO; DE MEJIA, 2008)			16.70	3.90	7.60	1.50	33.00	6.90			81.20	77.60
(DARTORA et al., 2011)			18.90	4.68	4.61	1.63	14.42	3.03				
(BORGES et al., 2019)			10.70	5.04	3.32	1.33	0.49	0.25				
(LORINI et al., 2021)							26.90	18.05			16.22	13.03
(PAGLIOSA et al., 2009)			16.00	3.40								
(ROSSA et al., 2017)	1.26	0.87									130.11	115.67
(STRASSMANN et al., 2008)			8.15	2.03	0.519	0.17					4.98	1.98
(DUARTE et al., 2022)	21.40	14.00	20.40	8.80	6.00	2.00	48.00	40.00				
(RAKOCEVIC et al., 2023)			14.50	7.00	3.50	1.20	26.00	10.90	0.68	0.12		
(SCHERER et al., 2002)			15.10	7.40	1.11	5.07						
(MARX et al., 2003)			18.72	11.60	11.64	7.05						
(CARDOZO et al., 2007)	8.15	3.15	3.15	6.67	2.37	0.37						
(NAKAMURA et al., 2009)			13.77	2.26	8.31	1.76	20.31	13.44				
(CARDOZO et al., 2010)			16.63	2.48	8.07	1.06	22.81	13.65	0.27	0.37		
(VALDUGA et al., 2016)			46.00	13.00	1.50	0.16	0.56	0.23				
(FRIEDRICH et al., 2017)			13.80	0.20	8.50	1.00	28.10	15.90				
(VIEIRA et al., 2021)			28.26	11.47	5.89	1.43					111.5	73.98

MxT, total methylxantines; C, caffeine; T, theobromine; ACG, chlorogenic acid ; AC, caffeic acid; CF, phenolic compounds. [1] mg g^{-1} of extract; [2] mg EAG g^{-1}, mg gallic acid equivalent (EAG) per gram of sample

Table 3. Summary of studies on the influence of each agronomic factor on the content of a specific secondary metabolite in *Ilex paraguariensis*.

Agronomic factor	MxT	C	T	ACG	ACQ	AC	CF	Reference
Seasonality	+							(SCHUBERT et al., 2006)
Seasonality				+				(BUTIUK et al., 2016)
Seasonality							+	(Ferrera et al., 2016)
> Leaf age		-	-					(ESMELINDRO et al., 2004)
> Leaf age			+					(STRASSMANN et al., 2008)
> Leaf age		*						(YIN; KATAHIRA; ASHIHARA, 2015)
> Leaf age	-	-					-	(BLUM-SILVA et al., 2015)
> Light intensity		-	-					(ESMELINDRO et al., 2004)
> Light intensity	-							(COELHO et al., 2007)
> Light intensity		+	+	+			+	(HECK; SCHMALKO; DE MEJIA, 2008)
> Light intensity		+	+	+				(DARTORA et al., 2011)
> Light intensity							-	(Ferrera et al., 2016)
> Light intensity	-							(Rossa et al., 2017)
> Light intensity		-		-				(BORGES et al., 2019)
> Light intensity		-						(TORMENA et al., 2020)
> Light intensity							+	(LORINI et al., 2021)
> Light intensity		+					+	(STRASSMANN et al., 2008)
Cultivation system		*						(PAGLIOSA et al., 2009)
Fertilization	*						*	(Rossa et al., 2017)
Morphotypes	*	+	*		*		*	(DUARTE et al., 2022)
Sexual dimorphism	+							(PAULI et al., 2019)
Sexual dimorphism		*	*	*		*		(RAKOCEVIC et al., 2023)
Genetic factor		+	+					(SCHERER et al., 2002)
Genetic factor		+	+					(MARX et al., 2003)
Genetic factor	+	+	+	+			*	(CARDOZO et al., 2007)
Genetic factor	+	+	+					(NAKAMURA et al., 2009)
Genetic factor	+	+	+	+				(CARDOZO et al., 2010)
Genetic factor		+	+	+				(VALDUGA et al., 2016)
Genetic factor	+	+	+			+		(FRIEDRICH et al., 2017)
Genetic factor		+	+				+	(VIEIRA et al., 2021)

MxT, total methylxantines; C, caffeine; T, theobromine; ACG, chlorogenic acid; AC, caffeic acid; CF, phenolic compounds; *, neutral influence; +, positive influence; - negative influence.

The studies found in the literature regarding the effect of luminosity on levels of bioactive compounds are divergent. Strassmann et al. (2008) reported higher caffeine levels in monoculture with 100% solar radiation. Dartora et al. (2011) and Heck et al. (2008) concluded that shaded planting in the forest reduced caffeine concentration in plants. However, Borges et al. (2019) found differences in caffeine, theobromine, and chlorogenic acid levels between shading levels, with varying results at the two study locations, making the shading effect inconclusive. Nevertheless, as discussed earlier, shading exceeding 70% seem to favor caffeine accumulation.

It is crucial to note that high shading levels decrease the plant's photosynthetic rate, reducing leaf biomass and leading to lower biomass productivity (Marques et al., 2014; Vogt et al., 2016; Westphalen et al., 2020; De Aguiar et al., 2022). Reduced photosynthesis lowers carbohydrate concentrations, affecting growth rate more than nutrient absorption. In response to adverse conditions, nutrients accumulate in tissues, producing defense compounds such as alkaloids (like methylxanthines) (Bryant et al., 2009; Aguiar, 2021). However, under extreme shading conditions, primary metabolite production, like sucrose, decreases. Sucrose provides a carbon skeleton for organic compounds and stores energy for chemical reactions, essential for secondary metabolite biosynthesis (Li et al., 2015). Another crucial point is that in different studies evaluating the effect of luminosity, the plantations were of seminal origin, which means that the genetic factor influenced the results due to the high heritability of methylxanthines (Cardozo et al., 2010; Nakamura et al., 2009; Benedito et al, 2023). More precise studies are necessary to assess the influence of the luminosity factor on the production of bioactive compounds in yerba mate, isolating the genetic effect, for example, using clonal genotypes.

In addition to methylxanthines, some studies have demonstrated the effect of luminosity on the production of phenolic compounds. Lorini et al. (2021) and Heck et al. (2008) observed higher concentrations of phenolic compounds in plants grown in full sun compared to shaded plants. Phenolic compounds are produced by plants in response to environmental stimuli (SHARMA et al., 2019). In full sun cultivation, plants are exposed to a much higher concentration of UV radiation, increasing photosynthetic rates but also causing plants to absorb other electromagnetic waves of higher energy, which can generate free radicals and induce cellular damage. To protect themselves, plants produce antioxidants, such as chlorogenic acid (Heck et al., 2008; Taiz et al., 2017).

Regarding the seasonality factor, Ferrera et al. (2016) observed a higher production of phenolic compounds in mate leaves during autumn compared to summer. Schubert et al. (2006) evaluated the production of methylxanthines in two populations throughout the year and observed an increase in concentration during summer for both populations. Butiuk et al. (2016) found a substantially higher content of chlorogenic acid at the beginning of the harvest (April and May) compared to the end of the harvest (September). These results indicate that the harvest season has a diverse effect on the production of different bioactive compounds in mate, and as it can alter the plant's chemical composition, it influences the quality of the raw material.

Environmental conditions can redirect plant metabolism, regulating the production of secondary metabolites (JAN et al., 2021); thus, the variation of bioactive compounds with seasonality is inevitable. According to Bhandari et al. (2019), plants produce secondary metabolites to tolerate stress situations resulting from environmental factors such as radiation, temperature, water deficit, salinity, etc. Therefore, more precise studies should be conducted to understand the role of seasonality in the content of bioactive compounds, expanding the possibilities of obtaining commercial products with concentrations of compounds according to consumer requirements.

Leaf age is an important factor that determines the optimal harvesting point for yerba mate, being highly related to the synthesis of secondary metabolites. Authors have identified that young mate leaves have higher levels of methylxanthines and chlorogenic acids than mature leaves (Blum-Silva et al., 2015; Esmelindro et al., 2004). Caffeine, a methylxanthine, plays a crucial role in direct defense against herbivorous insects (Li et al., 2020). Similarly, phenolic compounds, such as chlorogenic acids, are efficient in plant protection against herbivory, acting to reduce digestibility in herbivores (MOREIRA et al., 2018). Thus, the increased production of these compounds in young leaves is likely related to the plant's defense against insect and other predator attacks, as young leaves are the preferred plant organs for insects due to being more palatable and having less thick cell walls (COLEY; ENDARA; KURSAR, 2018).

The variation in bioactive compounds content in leaves due to leaf age (young or mature leaves) is related to the leaf development stage, easily defined for yerba mate by leaf coloration, where light green represents young leaves, and dark green characterizes complete leaf expansion (Aguiar et al., 2022). Generally, in conventional yerba mate production systems, only mature leaves are harvested. These results are relevant to the productive sector as they demonstrate the potential of young leaves for products focused on bioactive compounds, such as energy drinks or industrial compound extraction, for example. Thus, different field management practices could be implemented, such as reducing the harvest interval, and consequently, increasing the number of harvests, aiming for a higher proportion of young leaves. Additionally, this opens up possibilities for the development of new production systems, such as semi-hydroponic cultivation in a greenhouse, which has achieved high productivity and a large yield of young leaves, with the potential to obtain chemically more interesting raw materials for specific markets (Aguiar et al., 2022).

Fertilization is a relevant factor in the commercial biomass production of yerba mate, and some studies have been conducted on the influence of fertilizers, especially nitrogen-based ones, on the production of bioactive compounds in the species (Gabira, 2022; Tomasi, 2020). Nitrogen participates in the biosynthesis of methylxanthines through nucleotides containing the nitrogenous bases adenine and guanine, forming xanthosine, which is the initial substrate for the synthesis of methylxanthines such as caffeine and theobromine (YIN; KATAHIRA; ASHIHARA, 2015). Therefore, it is believed that the supply of this macronutrient favors the production of methylxanthines. Rossa et al. (2017) evaluated plants without fertilization, with conventional fertilization, and slow-release fertilization associated with different light levels and did not observe a clear influence of fertilization on the increase in total methylxanthin and phenolic compound levels.

Several studies have been conducted to assess the effect of sexual dimorphism on the production of bioactive compounds in mate. Since female reproductive investments tend to be greater than male investments due to seed and fruit production, the hypothesis is that there is a superiority in metabolite production in male plants (PAULI et al., 2019). This hypothesis was confirmed by Pauli et al. (2019), who identified higher levels of methylxanthines in male plants compared to female plants, regardless of the phenophase and light incidence. On the other hand, Rakocevic et al. (2023) suggest that the association between gender and the accumulation of bioactive compounds is apparently random, and new studies need to be conducted to demystify these responses.

A significant portion of the selected studies for this review investigated the genetic influence on the chemical composition of yerba mate, and all of them identified high variation in the levels of the studied compounds among progenies. These studies hold significance for genetic improvement programs, showcasing the high genetic diversity and enabling selection for specific traits, resulting in increased productivity and profitability (WENDLING et al., 2018; Benedito et al., 2023). The growing interest of industries in yerba mate and the species' potential for food, beverage, cosmetic, and pharmaceutical production make genotype characterization a crucial step for establishing profitable plantations with minimally controlled chemical characteristics.

Furthermore, yerba mate exhibits high morphological variation, both in natural populations and commercial plantations, primarily related to the size, hairiness, and color of leaves and stems (WENDLING et al., 2016). Plants with morphological similarities are generally referred to as morphotypes. This grouping of plants into morphotypes serves as an alternative selection criterion for genetic improvement, reducing evaluation time and costs, as it involves a visual selection methodology (WENDLING et al., 2016). Duarte et al. (2022) investigated the chemical composition of five yerba mate morphotypes – "sassafras," dark green, matte green, gray, and "furry" – all of which exhibited antioxidant capacity and high levels of phenolic compounds. The authors found differences only in the caffeine content between the dark green morphotype, with higher caffeine content, and the "furry" morphotype, with lower caffeine content.

3.2 RAW MATERIAL PROCESSING AND BIOACTIVE COMPOUNDS

In the literature, we identified 28 studies that explored the interrelation between the processing factors of yerba mate raw material and the content of bioactive compounds. These studies were conducted in Brazil (14) and Argentine (12) (Table 4).

These investigations are relatively recent, commencing from the early 2000s (Table 4), which can be justified by the growing interest of various consumer markets due to the significance and abundance of compounds found in yerba mate (BECKER et al., 2019). Many industrial sectors seek strategies to achieve higher levels of phenolic compounds, caffeine, and antioxidant capacity (BRACESCO, 2019). Therefore, understanding the chemical changes during the processing of yerba mate becomes essential, enabling targeted approaches for specific markets.

Table 4. Summary of published studies that correlated processing factors with bioactive compounds production in *Ilex paraguariensis*.

BIOACTIVE COMPOUND	PROCESSING FACTOR	RESULTS	REFERENCE	COUNTRY
ACG	Processing steps	During roasting, a significant loss in neo-ACG and ACG content occurred.	(BUTIUK et al., 2016)	Argentine
C, T e ACG	Processing steps	There was a decrease in the content of methylxanthines when processed by blanching and drying, however, they presented greater antioxidant capacity. The oxidized leaves showed a decrease in antioxidant activity.	(DARTORA et al., 2011)	Brazil
Taninns	Processing steps	Higher tannin content was observed in the stages after blanching, resulting from the high temperature applied in the process, and the low moisture content of the yerba mate in these stages.	(FRIZON et al., 2015)	Brazil
C, T e AC	Processing steps	The samples obtained after blanching, drying, and aging processes have a higher content of biologically active ingredients (caffeoil, caffeine, theobromine, and rutin) when compared to green leaves.	(ISOLABELLA et al., 2010)	Argentine

C, ACG, AC e CF	Processing steps	Blanching caused an increase in bioactive compounds when compared to drying in natura. High toasting temperatures caused a decrease in CF content.	(Riachi et al., 2018)	Brazil
C, T, AC e ACG	Processing steps	Aged yerba mate showed higher levels of theobromine, chlorogenic acid, caffeic acid, and caffeine than green yerba mate.	(SCHENK et al., 2021)	Argentine
C	Processing steps	Total caffeine loss averaged 30% (8% after bleaching; 8% and 13% after the first and second drying stages, respectively).	(SCHMALKO; ALZAMORA, 2001)	Argentine
C, AC, ACG e CF	Processing steps	The industrialization process reduced the total antioxidant activity of the extracts, but not the content of bioactive compounds.	(TURNER et al., 2011)	Argentine
CF	Processing steps	Pre-dried and aged yerba mate presented greater antioxidant capacity. Positive relationship between antioxidant capacity and phenolic compound content.	(Valerga et al., 2012)	Argentine
CF	Processing steps	Industrial processing modified the polyphenol composition and antioxidant activity of the yerba mate extracts. Pre-dried and dried/aged leaves were the most suitable raw materials.	(VALERGA; SHORTHOSE; LANARI, 2013)	Argentine
ACG e C	Processing steps	The results showed that the dried and ground leaves had higher levels of ACG (341.70 µg/mL) and caffeine (211.50 µg/mL).	(BASTOS et al., 2006)	Brazil
CF	Processing steps	The antioxidant capacity was higher in aged yerba mate (37.66%) when compared to dried yerba mate (27.65%).	(EFING et al., 2009)	Brazil
C e T	Drying methods	Increase in the content of compounds, such as caffeine and theobromine when drying the leaves in microwave.	(ESMELINDRO et al., 2004)	Brazil
C, T e ACQ	Drying methods	Products dried using wood burning to generate heat had higher total ACQ contents compared to air-dried products, while air-dried products contained more theobromine than products dried using wood burning.	(HECK; SCHMALKO; DE MEJIA, 2008)	Argentine
C, T, MXT, AC e CF	Drying methods	Microwaves can be an alternative drying technique, as the CF content, antioxidant capacity, MXT, and AC were similar or superior to conventional drying processes.	(Tomasi et al., 2021a)	Brazil

C, T, MXT, CF e AC	Drying methods	Overall, the microwave and freeze dryer were highly efficient with high CF, antioxidant capacity, C, and AC values.	(Tomasi et al., 2021b)	Brazil
CF	Drying methods	Samples dried with conventional hot air showed 47% lower CF content.	(ZANOELO; CARDOZO-FILHO; CARDOZO, 2006)	Brazil
C	Drying methods	Drying temperatures (60, 80, and 100°C) did not affect the caffeine content, however with an increase in drying time there was a 10% decrease in the content.	(RAMALLO; LOVERA; SCHMALKO, 2010)	Argentine
ACG	Drying temperature	The results showed that the effect of temperature is more important than processing time on reducing ACG content.	(BENINCÁ; KASKANTZIS; ZANOELO, 2009)	Brazil
C, T, MXT, AC e CF	Toasting	In general, there was a reduction in the content of caffeine, caffeoylquinic acids, and, in some genotypes, theobromine after the toasting process.	(DUARTE et al., 2020)	Brazil
ACG	Toasting	Throughout the roasting process, the GCA content of mate teas decreased.	(INADA et al., 2022)	Brazil
ACG e CF	Toasting	The total ACG content of the toasted mate was lower than that of the dry mate.	(LIMA et al., 2016)	Brazil
ACG e CF	Aging	The rutin and ACG contents were not influenced by the aging systems, while the caffeic acid content increased only in accelerated aging.	(DUTRA; HOFFMANN-RIBANI; RIBANI, 2010)	Brazil
C e CF	Aging	The concentration of caffeine and CF decreased after the three aging methods: air humidity and temperature control, temperature control, and naturally aged.	(HOLOWATY et al., 2014)	Argentine
C e CF	Aging	During natural aging, CF content decreased, and caffeine remained constant.	(HOLOWATY et al., 2016)	Argentine
CF	Oxidation	Air humidity had an influence on the oxidative process of yerba mate and its antioxidant capacity. The highest levels of CF and antioxidant potential were obtained with a relative humidity of 90%.	(MOLIN et al., 2014)	Brazil
AC, ACG e C	Commercial product x *in natura*	The extracts prepared with green material showed greater antioxidant capacity than the commercial product.	(ANESINI et al., 2012)	Argentine

MxT, total methylxantines; C, caffeine; T, theobromine; ACG, chlorogenic acid; ACQ, cafeoilquinic acids; AC, caffeic acid; CF, phenolic compounds.

During processing, the raw material goes through different stages, such as pre-drying, blanching or scalding, drying in a rotary dryer or on a conveyor belt, grinding, and, in the case of mate tea production, toasting at temperatures ranging from 180 to 215 °C (ISOLABELLA et al., 2010). The selected studies assessed the variation of bioactive compounds during each processing phase, as well as the influence of different temperatures or drying methods on the content of these compounds (Table 5).

Table 5. Summary of studies on the influence of each processing factor on the composition of a specific bioactive compound in *Ilex paraguariensis*.

Processing factor	MxT	C	T	ACG	ACQ	AC	CF	Tan	Reference
Drying method		+	+						(ESMELINDRO et al., 2004)
Drying method			+		-				(HECK; SCHMALKO; DE MEJIA, 2008)
Drying method		+	+		+		*		(Tomasi et al., 2021a)
Drying method		*	*				*		(Tomasi et al., 2021b)
Drying method							+		(ZANOELO; CARDOZO-FILHO; CARDOZO, 2006)
Drying temperature				-					(BENINCÁ; KASKANTZIS; ZANOELO, 2009)
Drying temperature		-							(RAMALLO; LOVERA; SCHMALKO, 2010)
Commercial product		+		+		*			(ANESINI et al., 2012)
Processing steps				-					(BUTIUK et al., 2016)
Processing steps	-	-	-	+					(DARTORA et al., 2011)
Processing steps								+	(FRIZON et al., 2015)
Processing steps		+	+	+		*			(ISOLABELLA et al., 2010)
Processing steps		+	+				+		(Riachi et al., 2018)
Processing steps		+	+	+		+			(SCHENK et al., 2021)
Processing steps		-							(SCHMALKO; ALZAMORA, 2001)
Processing steps		+	+	+		*	+		(TURNER et al., 2011)
Processing steps		+		+			+		(Valerga et al., 2012)
Processing steps		+		+					(VALERGA; SHORTHOSE; LANARI, 2013)
Bleaching							+		(NOMURA et al., 2022)
Grinding		+		+					(BASTOS et al., 2006)
Toasting	-	-	-	-	-		-		(DUARTE et al., 2020)
Toasting				-					(INADA et al., 2022)
Toasting				-					(LIMA et al., 2016)
Aging				*					(DUTRA; HOFFMANN-RIBANI; RIBANI, 2010)
Aging		-					-		(HOLOWATY et al., 2014)
Aging		*					-		(HOLOWATY et al., 2016)
Oxidation				-					(DARTORA et al., 2011)
Oxidation							-		(MOLIN et al., 2014)

MxT, total methylxantines; C, caffeine; T, theobromine; ACG, chlorogenic acid; ACQ, cafeoilquinic acids; AC, caffeic acid; CF, phenolic compounds; Tan, tanines; *, neutral influence; +, positive influence; -, negative influence.

In the initial phase of traditional yerba mate processing, leaves undergo enzymatic inactivation known as scalding (*sapeco*), in which plant tissue is subjected to high temperatures through direct fire for a few seconds, potentially leading to chemical changes (Riachi et al., 2018). Generally, in most selected studies, increases in the content of phenolic compounds and antioxidant capacity of yerba mate were observed after scalding (ANESINI et al., 2012; BASTOS et al., 2006; DARTORA et al., 2011; ISOLABELLA et al., 2010; RIACHI et al., 2018; TOMASI et al., 2021a; VALERGA; RETA; LANARI, 2012; VALERGA; SHORTHOSE; LANARI, 2013).

The antioxidant activity is primarily attributed to the phenolic compounds in the extract, which transfer electrons and form intramolecular hydrogen bonds (COLPO et al., 2016). Scalding is a rapid process aimed at inactivating enzymes responsible for the oxidation of phenolic compounds, such as polyphenol oxidases (PPO) and peroxidases (POD), contributing to their preservation and consequently allowing for greater antioxidant capacity (CHEN; MARTYNENKO, 2018; ISOLABELLA et al., 2010), justifying the observed results.

The influence of industrial processing on the maintenance or degradation of methylxanthines (caffeine and theobromine) has also been studied. Some authors have shown an increase in caffeine content during scalding and the drying processes of yerba mate leaves (BASTOS et al., 2006; DUARTE et al., 2020; ISOLABELLA et al., 2010; RIACHI et al., 2018; SCHENK et al., 2021; TURNER et al., 2011). High temperatures can lead to cell rupture (BASTOS et al., 2006), releasing compounds that were possibly conjugated (MARTINEZ et al., 2014). Moreover, elevated temperatures reduce the material's moisture, promoting an increase in soluble solids, resulting in a higher quantity of compounds dissolved in the extract (BASTOS et al., 2006; HECK; DE MEJIA, 2007). However, other studies have demonstrated a reduction in methylxanthine levels in these processing stages (Dartora et al., 2011; Schmalko & Alzamora, 2001; Tomasi et al., 2021a, b). A study on coffee showed that caffeine is thermostable (WEI et al., 2012), but its content may decrease with the reach of toasting temperature (185 ºC) (CASAL; BEATRIZ OLIVEIRA; FERREIRA, 2000). Thus, the reduction in caffeine content observed in some studies may be related to scalding process, where leaves are subjected to temperatures of up to 400 °C (TOMASI et al., 2021).

The high temperatures used in the enzymatic deactivation stages (scalding) and drying of yerba mate leaves also influence the oxidation of chlorogenic acid (ACG), as observed by Benincá et al. (2009). The authors state that in the scalding process, where high temperature is applied for a short period, ACG oxidation is complete; whereas, in the drying process, where the temperature is lower but drying time is longer, ACG oxidation is 60%, indicating that temperature effect is more important than the influence of processing time on ACG conversion, suggesting the need for the development of alternative post-harvest technologies for the production of ACG-rich products.

In scalding, the smoke released during roasting is absorbed by leaves and is known to contain polycyclic aromatic hydrocarbons (PAHs) (THEA et al., 2016; VIEIRA et al., 2010), which are compounds strongly associated with reactive oxygen species, tumor initiation activity, and inducing oxidative stress, making it unsafe for consumer use (CIEMNIAK et al., 2019). Seeking a methodology with lower risk and greater ease in drying yerba mate leaves, some authors have explored possible alternatives for leaf drying (ESMELINDRO et al., 2004; HECK; SCHMALKO; DE MEJIA, 2008; TOMASI et al., 2021a, 2021b; ZANOELO; BENINCA; RIBEIRO, 2011).

One potential alternative for leaf drying and maintaining bioactive compounds is the use of microwave oven, where Esmelindro et al. (2004) found higher quantities of bioactive compounds compared to traditional drying methods. Tomasi et al. (2021a,b) list the microwave drying process for yerba mate leaves as technically practical, fast, and efficient in maintaining compounds with antioxidant and stimulant capacities for various industrial sectors. The authors emphasize that the efficiency of microwave drying may be related to the reduced drying time and uniform temperature distribution. Another alternative method to avoid the use of smoke is hot air drying. Heck et al. (2008) observed higher theobromine levels and lower levels of caffeoylquinic acids in hot air drying compared to traditional smoke drying. Zanoelo et al. (2006), seeking an alternative method to smoke usage that is more energy-efficient than hot air drying, evaluated the levels of phenolic compounds in yerba mate leaves dried with superheated steam, obtaining satisfactory results in both energy savings and higher phenolic levels compared to air drying. A distinctive characteristic of superheated steam drying of yerba mate leaves, confirmed by the authors, is the oxygen-free atmosphere, which reduces oxidizing reactions, contributing to greater phenol retention and, thus, favoring antioxidant activity (BUTKEVICIUTE et al., 2022; KIM et al., 2021; ZANOELO; CARDOZO-FILHO; CARDOZO, 2006).

To obtain yerba mate tea, in addition to the pre-drying (bleaching), drying, and grinding stages, the raw material undergoes a toasting stage at temperatures ranging from 180 to 215 °C (DUARTE et al., 2020; INADA et al., 2022; LIMA et al., 2016). Authors have identified a reduction in chlorogenic acid (ACG) levels in the toasting process, stating that these decreases occur due to the high temperatures employed in toasting, which promote ACG degradation (INADA et al., 2022; LIMA et al., 2016).

Aging or maturation is a stage in yerba mate industrialization with the objective of forming aromatic compounds and changing the green color to yellow, which, although not appreciated by the Brazilian consumer, is a requirement of importing markets, such as Uruguay (DUTRA; HOFFMANN-RIBANI; RIBANI, 2010; HOLOWATY et al., 2016; SCHMALKO; ALZAMORA, 2001). This process can be done naturally, where the dried and aged raw material is stored in deposits for 6 to 24 months, or accelerated, through which temperature and humidity are controlled, reducing the period to 30 to 60 days.

Authors have reported that the content of bioactive compounds, such as chlorogenic acid (DUTRA; HOFFMANN-RIBANI; RIBANI, 2010) and caffeine (HOLOWATY et al., 2016; ISOLABELLA et al., 2010), seems not to be influenced by aging systems; however, they observed a reduction in the total phenolic compound content and claim that this reduction may be related to chemical reactions (isomerization, polymerization, and oxidation) of phenolic compounds that occur during aging (HOLOWATY et al., 2014, 2016). Laorko et al. (2013) stated that increases in temperature and storage time lead to an elevation in the kinetics of degradation of phenolic compounds. Thus, different aging systems may yield different results regarding the content of these compounds.

Table 6. Maximum and minimum concentrations of each compound found in the studies; values expressed in mg g^{-1} dry basis.

Reference	Bioactive compounds													
	MxT		C		T		ACG		ACQ		AC		CF	
	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min			Max	Min
(SCHMALKO; ALZAMORA. 2001)			1.80	1.33										
(ESMELINDRO et al.. 2004)*			159.44	65.93	1.38	0.40								
(ZANOELO; CARDOZO-FILHO; CARDOZO. 2006)													35.40	16.90
(BASTOS et al.. 2006)			1.51	0.71			2.44	0.59						
(HECK; SCHMALKO; DE MEJIA. 2008)			16.70	3.90	7.60	1.50	33.00	6.90	80.50	20.90				
(DUTRA; HOFFMANN-RIBANI; RIBANI. 2010)							0.23	0.18					36.08	34.88
(ISOLABELLA et al.. 2010)			16,00	9,00	3.60	3.30	20,00	18,00			0.35	0.32		
(RAMALLO; LOVERA; SCHMALKO. 2010)			11,00	9.50										
(DARTORA et al.. 2011)			18.90	4.68	4.60	1.63	14.40	3.03						
(TURNER et al.. 2011)			14.90	9.10			20.60	18.40			0.50	0.33	88.50	75.90
(ANESINI et al.. 2012)			13.50	9.10			20.70	18.40			3.30	3.30		
(Valerga et al.. 2012)							14.50	0.08			7.30	0.09	101,00	4.15
(VALERGA; SHORTHOSE; LANARI. 2013)							14.50	0.08			7.30	0.09		
(HOLOWATY et al.. 2014)			0.44	0.26									4.10	3.41
(MOLIN et al.. 2014)													10	4.95
(BUTIUK et al.. 2016)					80.80	45.80								
(HOLOWATY et al.. 2016)			0.95	0.91									9.91	9.14
(LIMA et al.. 2016)							132,00	15,00	135,00	15,00				
(Riachi et al.. 2018)			13.83	10.22	1,00	0.80							90,00	5,00
(DUARTE et al.. 2020)	19.64	0.38	19.29	0.09	6.09	0.09	41.73	5.52	81.47	23.32			111.27	53.18
(SCHENK et al.. 2021)			65.55	39.95	10.21	3.10	57.64	39.33			44.27	32.50	6.10	2.60
(Tomasi et al.. 2021a)			13.83	10.22	1,00	0.80							90,00	5,00
(Tomasi et al.. 2021b)			14,00	7,00	1.18	0.55							80,00	9,00
(NOMURA et al.. 2022)													46.03	5.03

MxT: total methylxanthines; C: caffeine; T: theobromine; ACG: chlorogenic acid; ACQ: total of 3-CQ, 4-CQ, and 5-CQ; AC: caffeic acid; CF: phenolic compounds. *mg g^{-1} extract

4. FINAL CONSIDERATIONS

Factors related to planting, management, harvesting, and processing are key elements to produce high-quality yerba mate raw materials. Therefore, new yerba mate crops should be planned and managed to achieve pre-established products, adding value to the final product. Additionally, the processing of raw materials should aim to maintain bioactive compounds levels. This review has shown that studies with this focus are still scarce and present discrepancies in results. Thus, we highlight the need to develop new research, with the isolation of factors, especially genetic ones, to provide more precise answers. The results of this literature review can assist in the development of new studies, aiming to obtain products with superior chemical quality, in accordance with the specificity of each consumer market.

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CHAPTER II - CONTENT AND YIELD OF YERBA MATE CLONES BIOACTIVE COMPOUNDS IN SEMI-HYDROPONIC SYSTEM WITH CRESCENT NITROGEN DOSES

ABSTRACT

Yerba mate (*Ilex paraguariensis* A.St.-Hil.) can provide many valuable phytochemicals such as methylxanthines, caffeine and theobromine, and caffeoylquinic acids (CQA or CGA – chlorogenic acids). It is necessary to establish cultivation protocols to meet the demand for raw materials with specific phytochemical profiles. In this study, we analyzed the content and yield of bioactive compounds in two clones of yerba mate submitted to increasing doses of nitrogen for two years in a semi-hydroponic cultivation system. The leaves were classified as young or mature and ground after drying in a microwave oven. The aqueous extracts were analyzed using an Ultra-Fast Liquid Chromatograph (UFLC). The yield was calculated by multiplying compound contents by leaf dry mass. Young leaves presented higher levels of virtually all compounds than mature ones. Clone EC40 showed higher levels of caffeine, 4-CQA, and 5-CQA, and a higher yield of all compounds, except for theobromine, when compared to EC22. Increasing nitrogen doses increased methylxanthine levels; however, the yield of compounds decreased with higher N levels due to reduced leaf mass. With the maximum productivity dose, 206 mg L^{-1} of N, the compound productivity reached up to 21 g m^{-2} $year^{-1}$ of caffeine and 126 g m^{-2} $year^{-1}$ of CQAs in clone EC40, and 14 g m^{-2} $year^{-1}$ of theobromine on EC22. These results demonstrate that the proposed cultivation system is viable for producing yerba mate for industrial extraction of bioactive compounds. Raw material with specific phytochemical characteristics can be obtained by clones and leaf types selecting.

Keywords: *Ilex paraguariensis*. Plant Nutrition. Methylxanthines. Caffeoylquinic acids. Chlorogenic acids.

1 INTRODUCTION

Ilex paraguariensis A.St.-Hil. is a tree native and cultivated in Brazil, Argentina, and Paraguay, and its leaves are widely consumed in much of South America (Cardozo and Morand, 2016; Bisognin et al. 2019). Yerba mate has great social, economic, and ecological importance, considered a main non-timber forest product in southern Brazil. In addition to traditional drinks – chimarrão, tererê, and mate tea – the species can be successfully used for pharmaceutical, cosmetic, and culinary purposes (HECK AND DE MEJIA, 2007; GODOY et al. 2013; ALKHATIB AND ATCHESON, 2017; BARBOZA AND CAZAL, 2018).

The search for healthier products with higher nutritional value and benefits to human health has been growing worldwide (CARDOZO AND MORAND, 2016; GAWRON-GZELLA et al. 2021; GERBER et al. 2022). Yerba mate stands out in this scenario because it is a source of several bioactive compounds present in its leaves, with more than 200 compounds detected (MELO et al. 2020), including amino acids, carbohydrates, vitamins, anthocyanins, carotenoids, methylxanthines, phenolic acids, fatty acids, flavonoids, among others (HECK; DE MEJIA, 2007; BLUM-SILVA et al. 2016; TOMASI et al. 2021). Due to these phytochemical characteristics, new forms of yerba mate consumption have aroused market interest, such as the application in encapsulated extracts and other industrialized products (BECKER et al. 2019; GAWRON-GZELLA et al. 2021; GERBER et al. 2022) in addition to tea, energy drinks, and cosmetic lines (BRACESCO 2019).

Among the compounds of interest in yerba mate are methylxanthines – caffeine and theobromine – and phenolic compounds, predominantly caffeoylquinic acids (CQA or CGA – chlorogenic acids); these compounds have attracted the attention of the world market due to their high concentration in the species leaves. Methylxanthines are classified as disease-preventing agents, such as diabetes mellitus, cardiovascular, and neurodegenerative diseases (OÑATIBIA-ASTIBIA et al. 2017). Caffeine is a stimulating compound that acts on the central nervous system and brings benefits such as enhanced energy, mental focus, learning and memory; this compound also assists in exercise performance, avoiding fatigue, increasing coordination, reducing the perception of pain and fatigue, and can favor weight loss (DE PAULA; FARAH 2019). Phenolic compounds are mainly responsible for the high antioxidant and anti-inflammatory activity of yerba mate (RIACHI; DE MARIA 2017; LIMA et al. 2016).

It is necessary to adapt the cultivation systems to obtain the required levels of bioactive compounds, maximize their benefits to human health and expand the species' applications. Using clones with phytochemical characteristics of interest adds value to the raw material and increases the suitability of the final product. To produce energy drinks, for example, clones selected for their high caffeine content could be used (AGUIAR et al. 2024). In addition, it is necessary to develop appropriate cultivation techniques to meet these demands (STRZEMSKI et al. 2021). The semi-hydroponic system of yerba mate cultivation, also called CEVAD greenhouse (high-density cultivation of yerba mate in a greenhouse), consists of growing yerba mate in a semi-hydroponic system, with successive harvests of leaves (AGUIAR et al. 2023). This system is commonly used to harvest propagules used in clonal silviculture (SÁ et al. 2018; VIEIRA et al. 2021); however, some studies have already highlighted its efficiency to produce young and mature leaves, with the potential to obtain phytochemically differentiated raw material (AGUIAR et al. 2022; AGUIAR et al. 2024; TOMASI et al. 2024; VIEIRA et al. 2021).

Some advantages of using the semi-hydroponic system are the higher environmental control and ease of managing growth factors, such as nutrition. Nitrogen (N) is the main nutrient required for plant growth (XING et al. 2019) and the most exported macronutrient when yerba mate is harvested in commercial plantations (33 to 37 g kg^{-1}) (OLIVA et al. 2014; SANTIN et al. 2019). In addition to its importance in leaf production and growth, N is part of the structure of molecules such as caffeine and theobromine (ASHIHARA et al. 2017), compounds of great interest in yerba mate. Determining the N requirement for the species and its influence on secondary metabolites synthesis is a current demand. Previous study by Tomasi et al. (2024) demonstrated the reduction of leaf biomass productivity at high doses of N in this system. Thus, optimizing the N dose used in this cultivation model can maximize biomass and bioactive compounds productivity, along with the possible reduction of costs and environmental impact caused by the overestimated use of nitrogen fertilizers.

Given the above, the objective of this study was to determine the contents and estimate the productivity per area of yerba mate bioactive compounds as a function of two clones and five increasing doses of nitrogen. The hypotheses raised were a) differences between clones, due to the genetic control of the analyzed compounds; b) higher levels of methylxanthines with increasing nitrogen dose, as they are nitrogenous compounds; c) lower productivity of compounds at higher nitrogen doses due to reduced leaf mass productivity in these treatments. This work brings results of productivity of yerba mate compounds per area, data of great relevance due to species potential for methylxanthines and caffeoylquinic acids industrial extraction.

2. MATERIAL AND METHODS

2.1 EXPERIMENT ESTABLISHMENT AND MANAGEMENT

The study was conducted between June 2016 and February 2019 at the Laboratory of Forest Species Propagation at Embrapa Florestas, located in Colombo – PR, Brazil. For this study, two clones were used – EC22 and EC40 – from a progenies and provenance trial established in 1997, in Ivaí, Paraná, Brazil (Wendling et al. 2018). The clones were selected within the Breeding Program for Yerba Mate at Embrapa Florestas, according to the caffeine content in mature leaves of the mother trees in the field, being EC22 decaffeinated (0.02%) and EC40 with high caffeine content (2.35%) (HELM et al. 2015).

The experiment was established in a semi-hydroponic system inside a plastic greenhouse without environmental control, planted in gutters filled with crushed stone for drainage, and medium-grained sand as a substrate (Attachment 1, 2 and 3). Details of this new yerba mate cultivation system can be consulted in previous studies (Aguiar et al. 2022; Aguiar et al. 2023; Aguiar et al. 2024; Tomasi et al. 2024). To study the influence of nitrogen (N) on the production of bioactive compounds, five increasing doses of N were tested, supplied in nutrient solution via dripping (Table 7). The other nutrients were provided in equal concentration in all treatments. Nutrition was applied three times a day, totaling 3.6 mm day^{-1} in autumn and winter and 5 mm day^{-1} in spring and summer.

Table 7. Total nitrogen (N), nitrate ($NO3^-$), ammonia (NH4+), NO3-/$NH4^+$ ratio and electrical conductivity (EC) of nutrient solutions supplied to *Ilex paraguariensis* A.St.-Hil. clones.

Treatment	Total N (mg L^{-1})	NO_3^- (mg L^{-1})	NH_4^+ (mg L^{-1})	NO_3^-/NH_4^+	EC (mS m^{-2})
1	114	64	50	1.28	2.21
2	206	156	50	3.12	2.47
3	380	243	137	1.77	3.00
4	760	433	327	1.32	3.76
5	1142	624	518	1.20	4.74

Leaf harvests were carried out approximately every two months to determine leaf yield, totaling six harvests in the first year and five in the second year. Leaf classification as young or mature followed the methodology of Aguiar et al. (2022) and Tomasi et al. (2024). After harvesting, the leaves were dried in a microwave oven for 5 minutes (TOMASI et al. 2021), and the dry mass was determined on a precision scale. Detailed methodology and leaf yield results are described in Tomasi et al. (2024).

2.2 DETERMINATION OF BIOACTIVE COMPOUNDS

The dried leaves were crushed until a fine and homogeneous powder (granulometry less than 1 mm) for the analysis. Each biological sample consisted of 25 plants (5 replicates of 5 plants). The aqueous extract was prepared with 10 mg of sample and 2 ml of ultrapure water, vortexed for 30 s. The extractions were performed in a Thermomixer® at 60 °C and 450 rpm for 1 h and subsequent filtration through a 0.22 mm membrane. Sample moisture was determined in an oven at 105 °C until constant weight, in triplicate, for later correction of the mass to determine compounds contents on a dry basis.

Compounds determination was performed on a Shimadzu® Ultra-Fast Liquid Chromatograph (UFLC), controlled by the LC Solution software and equipped with an automatic injector and UV detector (SPD-20A). To separate the compounds, a Shim-Pack CLC-ODS (M)® column (250 x 4.6 mm, Ø 5 µm) was used, with a Shim-Pack CLC G-ODS® pre-column (10 x 4, 0 mm, Ø 5 µm), both Shimadzu®. The conditions for separating the compounds present in the aqueous extract (20 µL of injection) were: 30 °C with a flow of 0.5 mL min^{-1} of eluent with mobile phase A (H_2O:acetic acid Alphatec® - 99.9:0.1 v/v) and B (Merck® acetonitrile - 100%). The wavelength used for compound detection was 280 nm (fixed). The gradient elution program was: 0-15 min (3% B), 15-20 min (3-20% B), 20-40 min (20% B), 40-45 min (20-30% B), 45-55 min (30%-100% B), 55-75 (100% B), 75-80 (100-3% B) and 80-95 (3% B).

The methylxanthines caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) were identified and quantified from analytical curves obtained with Sigma® standards between concentrations of 0 to 1.0 mg mL^{-1} and from 0 to 0.5 mg mL^{-1} for caffeine and theobromine, respectively. The quantification of 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), and 5-caffeoylquinic acid (5-CQA) was obtained from the analytical curve between concentrations of 0 and 10 mg mL^{-1} of 3-CQA. The results were expressed in mg of compound per gram of sample (mg g^{-1}) on a dry weight. Compound analyzes were performed in chemical triplicates.

2.3 STATISTICAL ANALYSIS

Data referring to bioactive compound contents were submitted to non-parametric statistics using the Kruskal-Wallis test and, when necessary, the Wilcoxon test for comparison between pairs of means ($p < 0.05$), followed by Principal Component Analysis (PCA). Based on the leaf dry mass yield determined in a previous study (Tomasi et al. 2024) and bioactive compound contents, the yield of each compound per cultivation area (g m^{-2}) was estimated. For this purpose, the average of the chemical triplicates and the five replicates of leaf mass (young and mature leaves separately) was used, multiplying the variables. Subsequently, the total annual production of the compounds was calculated by the sum of the productivity of young and mature leaves throughout the year. With this, we adjusted Mixed Generalized Linear Models (GLMM), with the factors nitrogen doses and clones considered as fixed effects and years of cultivation as a random effect; for variables that did not have a significant effect on the year of cultivation, GLM (Generalized Linear Models, *Gamma* distribution, identity link function) was employed.

3. RESULTS

3.1 BIOACTIVE COMPOUND CONTENTS

Clones differed significantly for caffeine, theobromine, methylxanthines, 4-CQA, and 5-CQA (Table 8). EC40 had higher caffeine contents, on average 2.3 times higher than EC22.

As for the theobromine, the EC22 showed content two times higher than the EC40. Regarding leaf types, higher levels of all compounds were found in young leaves for both clones.

Table 8. Bioactive compounds contents (mg g^{-1} on dry weight) in leaves of *Ilex paraguariensis* depending on the clones, EC22 and EC40, and leaf type, young and mature.

Compounds	Clone			
	EC22		EC40	
Caffeine	8.41 ± 0.44 B		19.48 ± 0.40 A	
Theobromine	11.53 ± 0.20 A		5.68 ± 0.13 B	
Methylxanthines	19.94 ± 0.45 B		25.16 ± 0.41 A	
3-CQA	17.17 ± 0.36 A		16.57 ± 0.33 A	
4-CQA	16.78 ± 0.21 B		17.74 ± 0.21 A	
5-CQA	43.61 ± 1.03 B		46.98 ± 0.98 A	
Total CQA	77.56 ± 1.42 A		81.28 ± 1.32 A	
	Leaf type			
	Young	Mature	Young	Mature
Caffeine	12.78 ± 0.67 a	4.04 ± 0.29 b	21.98 ± 0.66 a	16.98 ± 0.35 b
Theobromine	11.57 ± 0.27 a	11.48 ± 0.29 a	6.46 ± 0.14 a	4.90 ± 0.21 b
Methylxanthines	24.35 ± 0.66 a	15.52 ± 0.39 b	28.44 ± 0.63 a	21.88 ± 0.38 b
3-CQA	21.09 ± 0.47 a	13.25 ± 0.32 b	20.25 ± 0.44 a	12.89 ± 0.29 b
4-CQA	17.62 ± 0.29 a	15.93 ± 0.30 b	18.79 ± 0.31 a	16.67 ± 0.27 b
5-CQA	48.30 ± 1.48 a	38.91 ± 1.35 b	50.95 ± 1.43 a	43.01 ± 1.28 b
Total CQA	87.02 ± 1.92 a	68.10 ± 1.83 b	90.00 ± 1.80 a	72.57 ± 1.68 b

Mean ± standard error. Means followed by different letters indicate a significant difference by the Wilcoxon test ($p < 0.05$). Capital letters for comparison between clones; lowercase letters for leaf type comparison within each clone.

Principal Component Analysis (PCA) revealed the compounds that most contributed to separating the data were caffeoylquinic acids in dimension 1 and methylxanthines in dimension 2 (Figure 2). Factors N doses and harvests were not clearly distinguished by PCA, but there was a satisfactory separation for clones and leaf types. A clear separation in dimension 2 was noted for the clones, with clone EC40 located in the upper quadrant, especially in the direction of caffeine (Figure 2A). Regarding leaf type, young leaves tended towards the upper right quadrant, towards caffeoylquinic acids and caffeine (Figure 2B), confirming Kruskal-Wallis test results.

Regarding the effect of nitrogen doses, it was observed that the levels of methylxanthines, especially caffeine, increased with increasing N doses, especially with 1142 mg L^{-1} (Table 9). Contrarily, caffeoylquinic acids showed higher levels at lower or intermediate doses of N, until 380 mg L^{-1}.

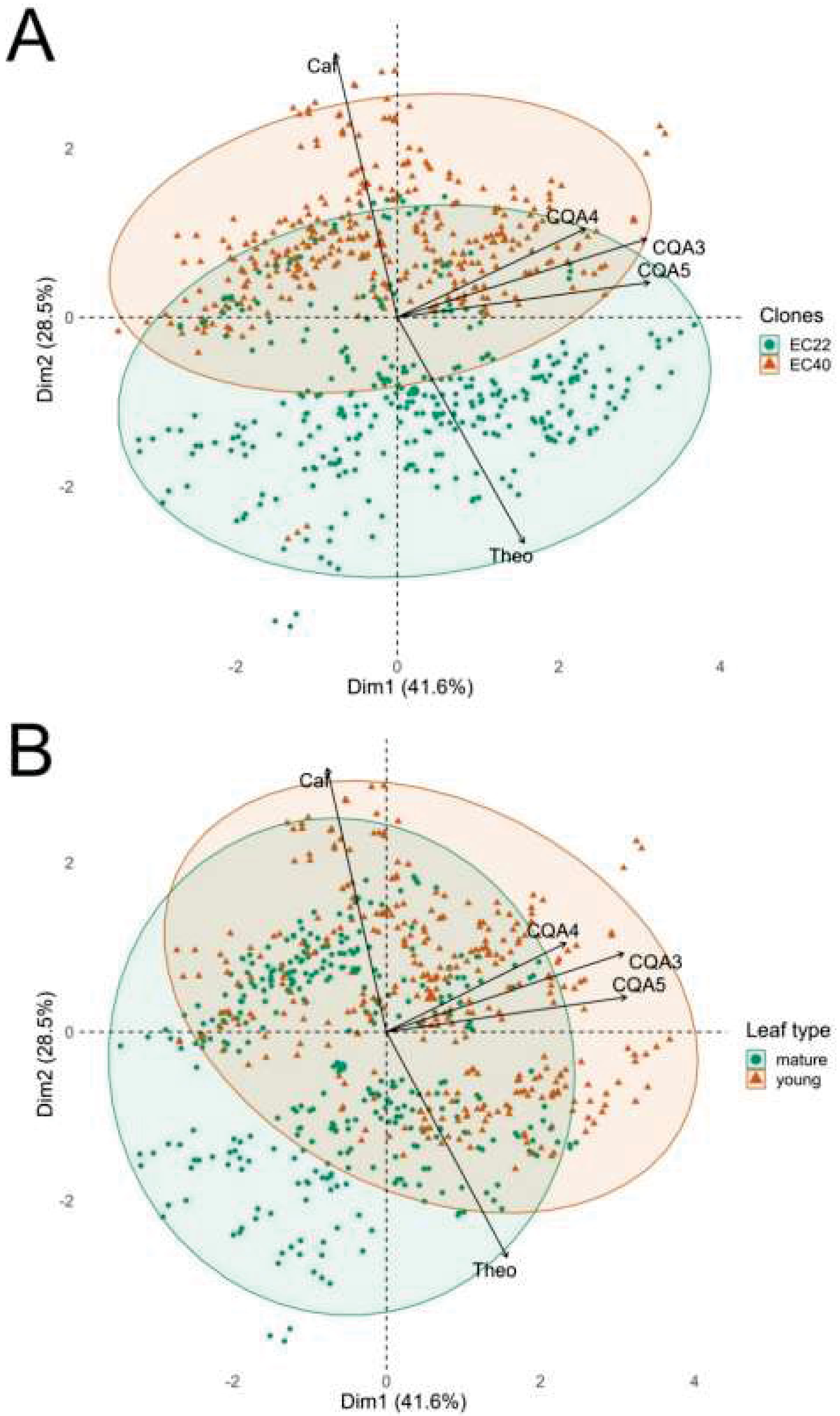

Figure 2. Principal Component Analysis biplot of bioactive compounds contents in *Ilex paraguariensis* leaves according to clones, EC22 and EC40 (A), and leaf types, young and mature (B). Compounds: Caffeine (Caf), theobromine (Theo), 3-caffeoylquinic acid (3-CQA).

Table 9. Bioactive compounds contents (mg g^{-1} on a dry weight) in leaves of *Ilex paraguariensis* clones, EC22 and EC40, depending on five nitrogen doses.

Compounds	Nitrogen dose (mg L^{-1})				
	114	206	380	761	1142
	EC22				
Caffeine	5.98 ± 0.87 c	7.80 ± 0.90 b	7.93 ± 0.95 b	8.15 ± 0.95 b	12.19 ± 1.08 a
Theobromine	10.01 ± 0.36 b	11.35 ± 0.33 a	11.80 ± 0.44 a	11.39 ± 0.41 a	13.09 ± 0.57 a
Methylxanthines	15.99 ± 0.96 c	19.15 ± 0.86 b	19.73 ± 0.93 b	19.54 ± 0.97 b	25.28 ± 1.02 a
3-CQA	17.55 ± 0.83 ab	19.48 ± 0.83 a	18.17 ± 0.78 a	15.45 ± 0.72 b	15.22 ± 0.72 b
4-CQA	16.38 ± 0.48 ab	18.07 ± 0.50 a	17.72 ± 0.48 a	16.05 ± 0.43 b	15.68 ± 0.43 b
5-CQA	42.93 ± 2.30 ab	48.55 ± 2.44 a	47.40 ± 2.40 a	40.74 ± 2.20 ab	38.41 ± 1.99 b
Total CQA	76.86 ± 3.21 ab	86.1 ± 3.32 a	83.29 ± 3.15 a	72.25 ± 2.98 b	69.32 ± 2.84 b
	EC40				
Caffeine	16.92 ± 0.80 c	17.81 ± 0.78 c	19.41 ± 0.98 bc	21.09 ± 0.85 ab	22.19 ± 0.85 a
Theobromine	5.18 ± 0.26 b	5.33 ± 0.23 a	5.53 ± 0.25 a	5.57 ± 0.25 a	6.78 ± 0.40 a
Methylxanthines	22.11 ± 0.82 d	23.14 ± 0.78 cd	24.94 ± 1.03 bc	26.66 ± 0.89 b	28.98 ± 0.82 a
3-CQA	16.68 ± 0.71 ab	18.70 ± 0.83 a	17.72 ± 0.80 ab	15.47 ± 0.67 bc	14.30 ± 0.55 c
4-CQA	18.35 ± 0.42 ab	18.07 ± 0.55 ab	18.81 ± 0.51 a	17.03 ± 0.48 bc	16.41 ± 0.36 c
5-CQA	50.09 ± 2.17 a	50.88 ± 2.36 a	48.23 ± 2.15 ab	43.70 ± 2.14 ab	41.97 ± 2.03 b
Total CQA	85.12 ± 2.81 ab	87.66 ± 3.14 a	84.77 ± 2.88 ab	76.21 ± 2.96 b	72.66 ± 2.60 b

Mean ± standard error. Means followed by different letters indicate a significant difference between N dose by the Wilcoxon test ($p < 0.05$).

3.2 COMPOUND YIELD PER AREA

We also analyzed the annual productivity of leaves (to understand the responses of the compounds), caffeine, theobromine, and CQAs. The year of cultivation (random effect) was only significant for CQAs, therefore, for this variable GLMM was used, and for all others, GLM was employed. Just theobromine productivity showed an interaction between nitrogen doses and clones (Table 10).

Table 10. ANOVA of GLM (fixed effects: nitrogen dose and clone) to leaf dry mass, caffeine, and theobromine annual productivity and GLMM (fixed effects: nitrogen dose and clone; random effect: cultivation year) to total caffeoylquinic acids annual productivity.

Source	Df	Dev.	Resid. Df	Resid. Dev.	F value	Pr(>F)
			Leaf dry mass			
Nitrogen	4	1.1152	15	0.8198	24.3081	3.90^{-5}
Clone	1	0.6062	14	0.2136	52.8557	2.69^{-5}
Nitrogen:clone	4	0.0981	10	0.1155	2.1375	0.1504
Residuals			19	1.9350		
			Caffeine			
Nitrogen	4	0.2296	15	8.3989	1.1110	0.4038
Clone	1	7.7999	14	0.5990	150.9987	2.34^{-7}
Nitrogen:clone	4	0.0604	10	0.5386	0.2924	0.8763
Residuals			19	8.6285		
			Theobromine			
Nitrogen	4	1.0203	15	1.3698	7.003	0.0059
Clone	1	0.3802	14	0.9896	10.438	0.0090
Nitrogen:clone	4	0.6134	10	0.3762	4.210	0.0297
Residuals			19	2.3902		
			CQAs			
	NumDf	DenDf	Sum SQ	Mean SQ	F value	Pr(>F)
Nitrogen	4	9	10260.0	2565.00	33.7334	1.99^{-5}
Clone	1	9	3034.6	3034.57	39.9089	0.0001
Nitrogen: clone	4	9	436.3	109.08	1.4346	0.2990

Df: degrees of freedom; Dev.: deviance; Resid.: residual; NumDf: numerator degrees of freedom; DenDF: denominator degrees of freedom; SQ: squares.

For CQAs, the first year showed higher productivity than the second year (Figure 3). The clone influenced leaf production, and all analyzed compounds. EC40 showed higher productivity of leaves, caffeine, and CQAs; while EC22 stood out for its high production of theobromine. The productivity of caffeine was 2.9 times higher in EC40 clone than EC22 at 206 mg L^{-1} N, reaching 21.24 (±1.51) g m^{-2} yr^{-1} at EC40. As for theobromine, the EC22 clone produced 1.8 times more than EC40, with up to 14.05 (±1.89) g m^{-2} $year^{-1}$ with 206 mg L^{-1} of

N. CQAs productivity reaching 126.36 (±5.45) g m^2 $year^{-1}$ for EC40 and 101.72 (±5.45) g m^2 $year^{-1}$ for EC22, both in the 1st year and 206 mg L^{-1} of N.

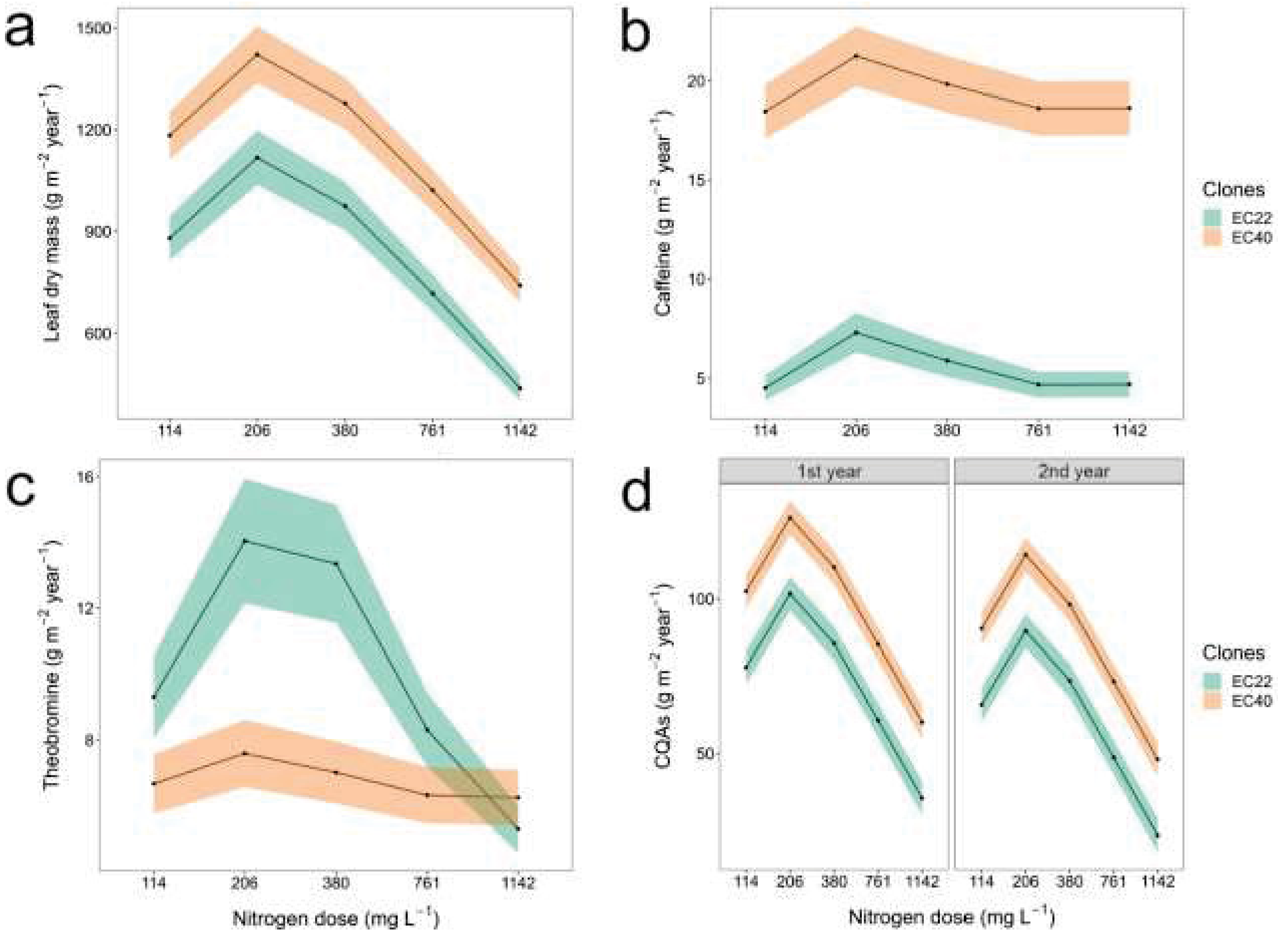

Figure 3. Annual productivity of leaves (a), caffeine (b), theobromine (c) and total caffeoylquinic acids - CQAs (d) as a function of *Ilex paraguariensis* clones, EC22 and EC40, and nitrogen doses. Productivity obtained by the sum of young and mature leaves.

The effect of N dose on annual productivity was significant for all variables, except caffeine. Thus, for caffeine, even with the drastic reduction in leaf productivity with the highest doses of N, caffeine productivity remained stable due to the greater accumulation of this compound in plants with higher N availability. In general, the dose of 206 mg L^{-1} of N resulted in greater productivity of leaves, and consequently, of the bioactive compounds analyzed. Higher doses of N decreased compounds productivity: for the EC22 clone caffeine productivity was 1.5 times lower in 1142 mg L^{-1} N compared to 206 mg L^{-1}, 2.6 times lower for theobromine, and 2.9 times lower for CQAs.

4. DISCUSSION

The results obtained in the present study are of great importance for cultivating yerba mate, especially concerning maximizing the production of raw material with specific compounds or for industrial extraction of these compounds. Young leaves showed higher levels of secondary metabolites; this fact can be attributed to the site of synthesis of these metabolites, since chlorogenic acids and methylxanthines are only synthesized in young tissues or have a higher concentration in young organs, such as expanding leaves (HERMS AND MATTSON, 1992; Yin et al. 2015). In *Camellia sinensis*, the protein profile was altered after leaf maturation, with a decrease in the PAL enzyme (phenylalanine amoniliasis), involved in the metabolic pathway of phenolic compounds (WU et al. 2019); it possibly occurs in other species, such as yerba mate. The higher concentration of secondary metabolites in young leaves is also in line with the "chemical defense theory," in which young leaves need higher protection against pathogens and herbivores and concentrate higher amounts of these compounds (ASHIHARA et al. 2008; RODZIEWICZ et al. 2014; YIN et al. 2015). Other studies with yerba mate also observed reduced caffeine, theobromine, and total phenolic compounds contents with leaf age (AGUIAR et al. 2024; BLUM-SILVA et al. 2015; ESMELINDRO et al. 2004).

The hypothesis of increasing methylxanthine contents with a higher supply of N was confirmed, especially for caffeine. It can be explained by the fact that methylxanthines are nitrogenous compounds formed from purine nucleotides in plants (ASHIHARA et al. 2017). While the methylxanthines were favored by the higher supply of N, the caffeoylquinic acids showed higher levels in low or intermediate N doses. The theory of Bryant et al. (1983) predicts that secondary metabolites with a carbon structure, such as phenolic compounds, are positively correlated with the carbon/nitrogen (C/N) ratio, and the opposite occurs for nitrogenous metabolites. Thus, in situations where there is no excess of fertilizers, for example, at the lowest N doses analyzed, there is a higher C/N ratio and, consequently, synthesis of secondary metabolites with a carbon structure. In situations of nutrient excess, the C/N ratio decreases and secondary nitrogenous metabolites accumulate. Thus, fertilization increases the concentrations of N-based secondary metabolites and decreases the concentrations of secondary metabolites with C skeletons (HERMS AND MATTSON, 1992). Studies with *C. sinensis*, another species recognized as a source of caffeine, also observed an increase in caffeine levels with a higher supply of N (BENTI et al. 2022; QIAO et al. 2018).

Confirming other hypotheses raised, the yield of all compounds per area decreased with increasing N availability due to lower leaf mass production under these conditions, except for caffeine, which did not have its productivity affected due to high contents in higher N doses. So, a 206 mg L^{-1} N dose is recommended to maximize the yield of leaves (TOMASI et al. 2024) and all analyzed compounds. Due to the low demand for this nutrient in this cultivation system, the production cost will be lower, as well as the environmental impact caused by the industrial production of this raw material (STRZEMSKI et al. 2021).

However, it is important to highlight that, regardless of the nitrogen dose applied, the clones maintained their methylxanthine profiles: EC40 with high caffeine and low theobromine content, and the opposite behavior for EC22. Aguiar et al. (2024) also observed a high influence of clone and leaf type on the contents of yerba mate bioactive compounds, not altering the phytochemical profile with the shading applied. This fact corroborates the high heritability for methylxanthines in *I. paraguariensis* (CARDOZO JUNIOR et al. 2010; NAKAMURA et al. 2009). Studies with *Coffea arabica* and *C. sinensis* also indicated that although the environment influences the caffeine content, it is mainly genetic factors that regulate the synthesis of this compound (BENTI et al. 2022; GONTHIER et al. 2011).

The higher accumulation of theobromine and lower caffeine content in the EC22 clone are relate to the metabolic route of these compounds, where theobromine is the direct precursor of caffeine (YIN et al. 2015). In *C. arabica* was observed the inactivity of the enzyme caffeine synthase in some genotypes, indicating a higher accumulation of theobromine because caffeine biosynthesis does not occur (SILVAROLLA et al. 2004). A caffeine degradation pathway to theobromine was also reported in *C. sinensis* (DENG et al. 2020; ZHU et al. 2019), a fact that possibly occurs in *I. paraguariensis*. Thus, for the EC22 clone, there may be a lower conversion of theobromine to caffeine or a higher catabolism of the latter compound. Negative correlations between caffeine and theobromine in yerba mate were also observed by Nakamura et al. (2009), Cardozo Junior et al. (2010), Friedrich et al. (2017), and Duarte et al. (2020).

The two clones analyzed have different phytochemical profiles and compounds yield and could have specific industrial applications. According to the caffeine content, mature leaves of EC22 clone would be considered low caffeine, while EC40 has high levels of this compound (SCHUHLI et al. 2019). Low caffeine raw material, such as mature leaves of EC22 clone, may be requested by people with concerns associated with caffeine consumption, children, or consumers who prefer milder products. EC40 clone was more productive for all compounds, except for theobromine, as it presented high levels of compounds combined with high leaf mass yield (TOMASI et al. 2024). Thus, planting genotypes similar to EC40 is recommended when the raw material is destined for markets that demand high caffeine concentrations, such as for energy drinks or the manufacture of capsules with antioxidant capacity, due to the higher productivity of caffeoylquinic acids. In this clone, with a 206 mg L^{-1} N dose, the compound yield on a dry weight, extrapolated to hectare (considering 70% of the productive area, discounting the space for corridors) would reach 149 (±10.57) kg ha^{-1} $year^{-1}$ of caffeine, 53 (±7.14) kg ha^{-1} yr^{-1} of theobromine and 884 (±38.15) kg ha^{-1} yr^{-1} of CQAs.

The high productivity of caffeoylquinic acids demonstrates the great potential of yerba mate for the industrial extraction of these compounds. The species is one of the best sources of CQAs found in nature (LIMA et al. 2016), with potential for industrial extraction due to the high levels and few interfering substances in the extraction (BUTIUK et al. 2016). However, in contrast to other polyphenol-rich plants such as tea or coffee, research, and industry have yet to explore the potential of yerba mate to promote human health (Cardozo Junior and Morand 2016). The high concentration (until 9% on a dry weight basis) and yield (126.36 g m^2 $year^{-1}$) obtained in the present study confirm the great potential of yerba mate as a source of caffeoylquinic acids, crucial for the manufacture of antioxidant capsules, for example. In addition, we emphasize that there are still many other compounds with already proven bioactive effects in the species, such as some saponins (NAGATOMO et al. 2022; PUANGPRAPHANT et al. 2011; PUANGPRAPHANT AND DE MEJIA 2009) that may also be of industrial interest.

5. CONCLUSION

The bioactive compounds content and yield proved to be highly dependent on the genotype. Knowledge of each genotype's phytochemical characteristics allows for more suitable cultivation for industrial purposes, enabling the development of new yerba mate products with high added value. Young leaves have higher levels of all bioactive compounds than mature ones, being a promising font of methylxanthines and caffeoylquinic acids. Nitrogen fertilization increases the levels of methylxanthines; however, the yield of phytochemicals decreases at doses greater than 206 mg L^{-1} N. The semi-hydroponic system provides a high yield of bioactive compounds from yerba mate, especially caffeoylquinic acids; this cultivation system is recommended to produce leaf mass for compounds' industrial extraction.

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FINAL CONSIDERATION AND FUTURE RESEARCH PERSPECTIVES

This research sought to explore and understand comprehensively the effects of agronomic and processing factors on the quality of yerba mate (*Ilex paraguariensis*) raw material, aiming to offer substantial contributions to what we already know about this topic. The literature review (first chapter) showcased the scarcity of studies focusing on this area, in addition to divergences in the results found. Therefore, we highlight the urgency of conducting new researches, emphasizing factors isolation, especially genetic ones, to offer more accurate answers. The second chapter made evident the potential of the semi-hydroponic cultivation system to yerba mate, clarifying that genetic factor plays a determining role in the selection of potential materials for bioactive compounds production. Furthermore, it was observed that the use of clonal materials facilitates researches aimed at the study of other factors, providing reliability to results through genetic factor isolation.

Therefore, the results achieved in this thesis have the potential to guide the onset of new researches, aiming the development of products with superior chemical quality, aligned to the specific demands of each consumer market. Furthermore, future studies are extremely important to evaluate the kinetic parameters of N absorption to verify the preference of nitrogen absorption (nitrate or ammonia) by yerba mate.

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ATTACHMENTS

Attachments 1 – Gutters Dimensions

Attachments 2 – Greenhouse without environmental control and introduction of plants in semi hydroponic system

Attachments 3 – Leaf mass production