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Use of cytogenetic techniques and flow cytometry to characterize the yacon genome

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ABSTRACT. Yacon (*Smallanthus sonchifolius*), a tuberous root native to the Andean region, is cultivated in various countries and is valued as a functional and medicinal food due to its nutraceutical properties. These properties include probiotic, antidiabetic, antioxidant, and antimicrobial effects, which contribute to its beneficial impact on human health. Despite this, the genetic diversity of yacon, which significantly influences trait expression and phenotypes, remains poorly explored. To date, only the number of chromosomes in the karyotype has been reported. This study, therefore, aimed to measure the nuclear genome size, determine the chromosome number, and characterize the karyotype of different yacon genotypes. For the first time, this research presents the karyogram and calculates the 2C nuclear value of yacon genotypes cultivated in a tropical environment outside the Andean region. This analysis is particularly significant as genetic material can be influenced and altered by environmental conditions. Our findings aim to identify such potential variations. The karyotype was characterized by 29 pairs of chromosomes: 13 submetacentric (1–5, 7–12, 14, 20) and 16 metacentric pairs (6, 13, 15–19, 21–29), with sizes ranging from 3.64 to 1.67 µm and secondary constriction in chromosome pair 21. Moreover, the karyotype presented a chromosome number of 2n = 58, contrasting with individuals of yacon cultivated in tropical environments, which presented a chromosome number of 2n = 32. The average 2C DNA content found was 2C = 6.39 pg, corroborating previous reports that presented relative variations from 2C = 5.82 to 6.12 pg. The observed genome 1C = 3,124.710 Mbp was considered small. Our methodology successfully obtained prometaphase and metaphase chromosomes, free of cytoplasmic traits and without overlaps. This study enriches the fundamental genetic knowledge of yacon, contributing to its conservation, evolution, and diversification, and affecting its agricultural potential beyond the Andean regions.

Keywords: Asteraceae; chromosome number; karyotype; *Smallanthus sonchifolius*; 2C nuclear value.

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Introduction

Yacon (*Smallanthus sonchifolius* [Poepp. & Endl.] H. Rob.), a perennial herbaceous plant native to the Andes, is typically cultivated in an annual cycle (Gurung et al., 2018). Historically integrated into Andean agriculture since pre-Columbian times, this crop thrives at altitudes ranging from 1,800 to 2,800 meters (Szokalo et al., 2020). Its cultivation has expanded globally to countries including Brazil, Japan, the Czech Republic, the United States, New Zealand, Italy, and Germany (Ojansivu et al., 2011; Kamp et al., 2019). This rising international interest in yacon stems from its value as a functional food, rich in beneficial compounds that have garnered significant attention from the food and pharmaceutical industries (Gusso et al., 2015; Silva et al., 2018).

As a dietary supplement, yacon aids in preventing and treating chronic conditions such as colon cancer, high cholesterol, and obesity (Caetano et al., 2016). It is rich in fructooligosaccharides and phenolic compounds, which exhibit antioxidant (Valentova et al., 2003; Oliveira et al., 2017) and antidiabetic properties (Leidi et al., 2018). Additionally, yacon dry leaf extract displays various phytochemical activities, including antimicrobial (Cruz et al., 2020), anti-inflammatory (Ambriz et al., 2016), and antioxidant effects (Cruz et al., 2019).

These health benefits have spurred increased commercial demand for yacon, extending its cultivation beyond traditional Andean regions (Ueda et al., 2019). This expansion introduces adaptive challenges, as plants must now thrive under new environmental conditions and cope with diverse biotic and abiotic stresses. Consequently, developing and selecting adapted genotypes become crucial, thus necessitating a deeper understanding of the yacon genome (Singh et al., 2019; Wagner et al., 2019).

Research indicates that yacon is an allopolyploid, derived from hybridization between *Smallanthus macroscyphus* (2n = 28, with a basic chromosome number of x = 7) and *Smallanthus riparius* (2n = 32, with a basic chromosome number of x = 8) (Viehmannová et al., 2009; Mansilla et al., 2010). This hybridization accounts for yacon's complex chromosomal configurations, including octoploid (2n = 6A + 2B = 58) and decaploid (2n = 9A + 3B = 87) structures, where 'A' denotes chromosomes from *S. macroscyphus* and 'B' from *S. riparius*.

Despite being vegetatively propagated, yacon exhibits significant chromosomal diversity (Grau & Rea, 1997). The most recent studies have identified chromosomal counts of 2n = 58 in Bolivia, 2n = 87 in Peru, 2n = 58 and 116 in Ecuador, and 2n = 30 and 32 in combined samples from Brazil and the USA (Svobodová et al., 2013).

Current knowledge of the yacon karyotype primarily focuses on chromosomal counts. Limited cytogenetic research has been conducted to delineate the chromosomal profile of yacon using classical cytogenetic techniques (Fernández et al., 2000). Additionally, some reported chromosome numbers should be approached with caution due to the absence of cytogenetic methodologies or the lack of referenced data to support these findings (Svobodová et al., 2013).

Karyotype characterization provides essential information about a species' genome and underpins the application of banding techniques and molecular cytogenetics. Chromosomal number and morphology are crucial for cytotaxonomy and can reveal insights into levels of ploidy (euploidy) and dysploidy (numerical and/or structural chromosomal rearrangements) (Li et al., 2021). Therefore, characterizing the karyotype enhances plant classification, understanding of taxonomic and evolutionary relationships among species, and elucidation of processes leading to diversification and speciation, which in turn aids in deciphering the origin and phylogenetic relationships (Medeiros et al., 2017).

Many species exhibit intraspecific variation (cytotypes) in chromosome number and ploidy level (Zonneveld, 2021), which also influences variations in nuclear DNA content. Nuclear C-value can diagnose and measure genomic differences among same-species individuals. In this sense, taxonomic, evolutionary, and phylogenetic studies, as well as breeding programs, have addressed nuclear C-value and karyotype data.

The nuclear C value, representing the total DNA content in the nucleus measured in picograms (1 pg = 10^{-12} g) or pairs of megabases (1 pg = 978 Mbp), is a crucial diagnostic tool for assessing genomic differences within a species (Kreplak et al., 2019). This measurement, often conducted via flow cytometry, has recorded yacon nuclear C values ranging from *1C* = 2.905 to 3.06 pg (Doležalová et al., 2004) and 1C = 2.980 to 3.005 pg (Valentová et al., 2006). To date, no cytogenetic studies have been reported on yacon cultivated in Brazil, highlighting a need for mapping chromosomal numbers and nuclear C values in the species grown domestically, thus enriching karyotypic characterization and cytotaxonomic studies.

Based on the above, this study focuses on measuring the size of the nuclear genome, determining chromosome numbers, and characterizing the karyotype of various yacon genotypes.

Material and methods

Biological material

A total of 44 yacon genotypes were collected from the in vivo collection at the Center for Agrarian Sciences and Engineering of the Federal University of Espírito Santo (coordinates: 20.3155° S, 40.3128° W; altitude: 30 m). Germplasm samples were sourced from farms across the primary producing municipalities of Espírito Santo State, Brazil, including Santa Maria de Jetibá (coordinates: 20.0258° S, 40.7439° W; altitude: 720 m), Afonso Cláudio (coordinates: 20.0774° S, 41.1261° W; altitude: 648 m), Santa Teresa (coordinates: 19.9362° S, 40.5979° W; altitude: 655 m), Santa Leopoldina (coordinates: 20.0981° S, 40.5273° W; altitude: 115 m), Domingos Martins (coordinates: 20.3603° S, 40.6591° W; altitude: 542 m), and Venda Nova do Imigrante (coordinates: 20.3278° S, 41.1350° W; altitude: 833 m).

Yacon seedlings were propagated from rhizophores (stem-like underground parts) and cultivated in pots containing sand, soil, and organic compounds mixed in equal parts. These pots were maintained in a

greenhouse with alternate-day irrigation to keep substrate moisture near field capacity, around 30-35% volumetric water content. Upon the development of young leaves and root systems, 20 representative plants were selected at random for nuclear DNA content measurement and karyotype characterization.

Nuclear-*C* value measurement

Flow cytometry parameters were set for *Smallanthus sonchifolius* samples and standard species using *Pisum sativum* (2C = 9.16 pg) and *Solanum lycopersicum* (2C = 2.00 pg) as internal standards (Praça-Fontes et al., 2011). Leaf fragments of *S. sonchifolius* (2 cm^2) and a reference species were placed into Petri dishes, minced (Galbraith et al., 1983) in 500 µL of OTTO-I nuclear extraction buffer (0.1 M citric acid, 0.5% Tween 20, 50 µg mL^{-1} RNAse, pH = 2.3) (Otto, 1990). After 3 minutes, an additional 500 µL of buffer was added, and the suspensions were filtered through 30 µm nylon membranes into 2 µL microtubes and centrifuged at 100 × g for 5 minutes. The supernatant was discarded, and the pellet was resuspended and incubated for 10 minutes in 100 µL of OTTO-I (Clarindo & Carvalho, 2009; Silva et al., 2018). Staining involved 1.5 µL of OTTO-II (Otto, 1990) containing 75 µM propidium iodide (Sigma®), 2.0 mM dithiothreitol (Sigma®), and 50 µg mL^{-1} RNAse, left for 30 minutes in the dark and filtered through 20 µm nylon membranes (Praça-Fontes et al., 2011). Nuclear suspensions were analyzed using a BD Accuri™ C6 flow cytometer (Accuri Cytometers, Belgium) equipped with a 488 nm 50 mW laser, two light scatter detectors (forward and side; FSC and SSC), and four fluorescence detectors. The average nuclear C value of the samples was determined in picograms by analyzing histograms with FlowMax® (Partec) software.

Karyotype characterization

Roots from yacon plants grown in pots were detached, rinsed under running water, and incubated with 3, 4, or 5 µM amiprofos-methyl (Sigma®) containing 0.3% dimethyl sulfoxide (DMSO, Sigma®). Incubation durations were 4, 6, 8, 12, 14, 18, and 24 hours at a refrigerated 5°C. Afterward, the roots were cut into approximately 1.5 cm segments, washed thrice with distilled water for 10 minutes each, and fixed in a methanol-acetic acid (3:1, v/v) solution, undergoing three solution changes before storage at -20°C.

Enzymatic maceration of the root meristems was conducted at 36°C for 2 hours in an enzymatic mixture consisting of 4% cellulose Onozuka R10 Yakult, 2.0% Sigma® C1184 cellulose, 0.4% Sigma® H077 hemicellulase, 0.5% macerozyme Onozuka R10 Yakult, and 1% Sigma® pectinase, diluted in a citrate buffer (10 mM citric acid + 10 mM tribasic sodium citrate, pH 4.8) at a 15:100 ratio (enzyme mixture to buffer). After another wash in deionized water, the roots were fixed again in methanol and acetic acid (3:1) and stored at -20°C (Silva et al., 2018).

Slides were prepared using cell dissociation and air-drying techniques, stained with 5% Giemsa (Merck®), and analyzed on an Olympus™ BX-60 trinocular photomicroscope. Prometaphase, metaphase, and anaphase stages were imaged using a 12-bit CCD Olympus® DP71 digital video camera attached to the microscope. Beyond chromosome count, yacon chromosome morphometry was assessed by arm length (µm) following the methods of Levan et al. (1964) and revised by Guerra (1986).

Results and discussion

Histograms revealed G_0/G_1 peaks in the samples and standards with coefficients of variation below 5%, indicating that the suspensions contained intact, isolated, and stoichiometrically stained nuclei. The mean 2C nuclear DNA content of yacon was measured at 6.390 ± 0.0017 pg, with 1C equivalent to 3,124.710 Mbp. According to Pellicer and Leitch (2020), this positions yacon's genome as small in the context of angiosperms, which categorizes genomes ranging from 2.8 pg < 2C ⩽ 7.0 pg as small.

This genomic size is notable because it significantly impacts gene and genome dynamics, including gene expression, replication, and mutation rates (Dodsworth et al., 2015), as well as broader physiological and developmental aspects of plants such as growth rates and reproductive cycles. From a wider perspective, genome size can significantly influence how plants grow, interact, and evolve, as well as their ability to handle stress, adapt to environmental changes, and coexist with other species (Moreira et al., 2024); however, differences between large and small genomes are still poorly understood (Pellicer et al., 2018).

Smaller genomes, like that of yacon, may afford advantages in adopting life cycle strategies crucial for agricultural expansion and adaptation to varied climates, a trait supported by Carvalho et al. (2021), who noted yacon's resilience to thermal and water stress, a crucial characteristic for Andean plants to thrive under tropical conditions.

The observed 2C value (6.390 ± 0.0017 pg) slightly exceeds previous reports by Doležalová et al. (2004) and Valentová et al. (2006), which ranged from 5.81 to 6.12 pg and 5.96 to 6.01 pg, respectively. In this study, internal standards *Pisum sativum* (2C = 9.16 pg) and *Solanum lycopersicum* (2C = 2.00 pg) were employed, deemed suitable for flow cytometry due to their genome sizes comparable to yacon (Praça-Fontes et al., 2011; Doležel et al., 2021). The discrepancies in nuclear DNA content may arise from interspecific variations, a key factor in plant speciation potentially preceding phenotypic differences (Huang, 2016; Huang et al., 2016; Fernández et al., 2017; Motsa et al., 2018; Žiarovská et al., 2019).

An 18-hour treatment of yacon roots with 3 µM amiprophos-methyl and 0.3% dimethyl sulfoxide at 5°C resulted in a higher metaphase index, producing chromosomes with distinct, well-preserved centromeres and telomeres without overlaps (Figure 1C). Conversely, other treatments, particularly those at room temperature, yielded fewer measurable chromosomes and exhibited increased chromatin compaction and overlap.

For the first time, yacon chromosomes were characterized by karyotypes (Figure 1) and karyograms (Figure 2), confirming a chromosome count of 2n = 58. This count corroborates earlier findings by Ishiki et al. (1997), supporting the hypothesis that yacon is an allopolyploid originating from the crossbreeding of *Smallanthus macroscyphus* and *Smallanthus riparius* (Ishiki et al., 1997). These findings align with reported ploidy levels and chromosomal data from various yacon germplasms, underscoring the genetic consistency observed across studies (Fernández et al., 2000; Viehmannová et al., 2009).

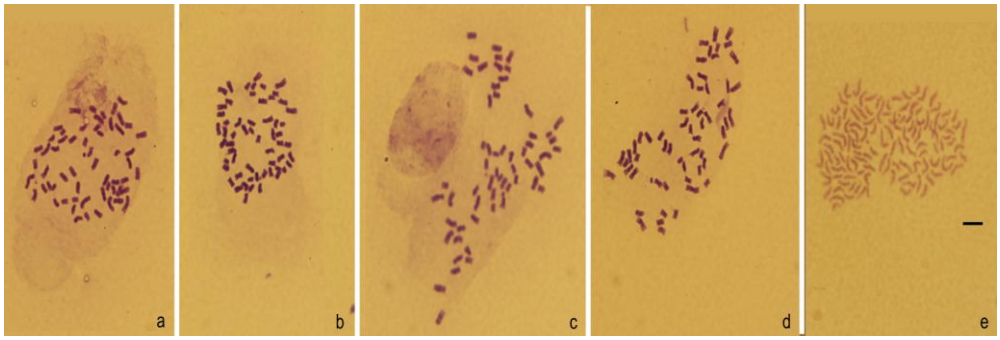

Figure 1. Chromosomes of yacon karyotypes at varying chromatin compaction levels with clearly defined centromeres and telomeres. These chromosomes were extracted from apical meristems of yacon roots, and treated with 3 µM amiprophos-methyl and 0.3% dimethyl sulfoxide for 18 hours at 5°C. Panels (a-d) depict metaphase with 2n = 58 chromosomes, and panel (e) shows anaphase with 2n = 116 chromosomes. Scale bar = 5 µm.

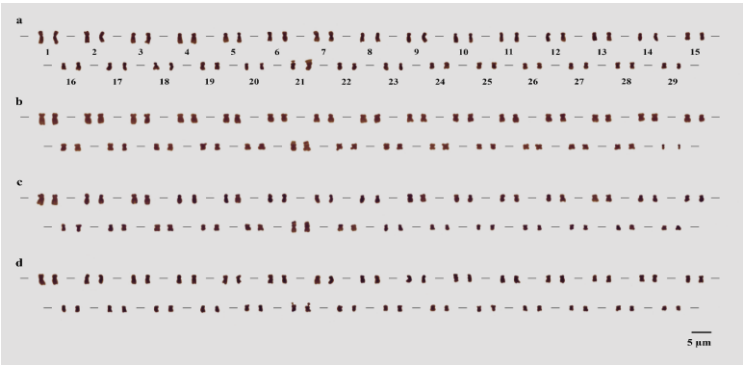

Figure 2. Yacon karyograms from metaphase chromosomes featuring well-defined primary constrictions. The karyograms display 2n = 58 chromosomes, consisting of 29 pairs: 13 submetacentric and 16 metacentric chromosomes. Notably, the short arm of chromosome 21 exhibits a secondary constriction.

Yacon may be an allopolyploid, composed of genomes from *S. macroscyphus* (2n = 28, A = 7) and *S. riparius* (2n = 32, B = 8), as proposed by Ishiki et al. (1997). The allopolyploid evolutionary origin of *S. sonchifolius* "yacon" (2n = 58 chromosomes) may have involved the duplication (euploidy) of the chromosome sets from these two ancestral species, potentially leading to autotetraploid species. The speculated cross involved a reduced reproductive cell from *S. riparius* and a non-reduced cell from *S. macroscyphus*, resulting in hexaploid offspring. Subsequent crosses between these hexaploids and non-reduced cells from *S. macroscyphus* (Mansilla et al., 2010). This origin could explain the prevalent octoploid karyotype of 6A (from *S. macroscyphus*) + 2B (from *S. riparius*) in vegetatively propagated yacon plants with 2n = 58 chromosomes

(Manrique et al., 2014; Ibañez et al., 2017). This karyotype was consistently observed in yacon plants from various Andean countries (Ecuador, Peru, and Bolivia) grown in the Czech Republic (Svobodová et al., 2013).

Earlier reports, such as those by Heiser (1963) in Ecuador and Talledo and Escobar (1996) in Peru, noted chromosome numbers of 2n = 60, suggesting a tetraploid structure. Further complexity is seen in reports of hexadecaploids (2n = 116) from *in vitro* studies (Viehmannová et al., 2009) and dodecaploids (2n = 87) in Brazilian materials (Manrique et al., 2014), highlighting the considerable chromosomal diversity in yacon despite its vegetative propagation (Grau & Rea, 1997).

The variability in yacon chromosome numbers highlights the presence of distinct cytotypes, critical for commercial cultivation. Ongoing research into genotypes better adapted to environmental conditions and thorough genetic data analysis are essential for advancing crop development and improving breeding programs (Lira et al., 2017). Numerous studies have reported various pests and diseases affecting yacon production (Lee et al., 2015), including occurrences in Brazil (Silva et al., 2015; Moraes et al., 2017; Camara et al., 2020; Oliveira et al., 2020).

Despite findings by Lorenzoni et al. (2017) of high genotypic similarity in yacon, variations in ploidy levels suggest a need for introducing new genotypes. This strategy could enhance genetic diversity and reduce the impact of biotic and abiotic stressors.

Morphometric analysis of the yacon karyotype reveals 29 pairs of chromosomes, comprising 13 pairs of submetacentric chromosomes (M – 1 – 5, 7 – 12, 14, 20) and 16 metacentric chromosomes (SM – 6, 13, 15 – 19, 21 – 29). The sizes of these chromosomes range from 3.64 µm (chromosome 1) to 1.67 µm (chromosome 29), leading to a karyotypic formula of 32 M + 22 SM. Notably, a secondary constriction is present on chromosome pair 21 (Figure 2 and Table 1).

Table 1. Morphological characterization of 2n = 58 of myotic chromosomes of yacon, based on Figure 2.

Chromosome		Arm				Related
No.	Total (µm)	Short	Long	r	Class*	Comp. (%)
1	3.647	1.382	2.265	1.64	SM	7.72
2	3.331	1.301	2.029	1.56	SM	7.05
3	3.191	1.140	2.051	1.80	SM	6.75
4	3.022	1.103	1.919	1.74	SM	6.39
5	2.926	1.074	1.853	1.73	SM	6.19
6	2.853	1.154	1.699	1.47	M	6.04
7	2.831	1.096	1.735	1.58	SM	5.99
8	2.735	1.015	1.721	1.7	SM	5.79
9	2.699	1.074	1.625	1.51	SM	5.71
10	2.669	0.978	1.691	1.73	SM	5.65
11	2.654	0.993	1.662	1.67	SM	5.62
12	2.588	0.993	1.596	1.61	SM	5.48
13	2.478	1.029	1.449	1.41	M	5.24
14	2.449	0.949	1.500	1.58	SM	5.18
15	2.360	1.037	1.324	1.28	M	4.99
16	2.346	0.971	1.375	1.42	M	4.96
17	2.287	0.978	1.309	1.34	M	4.84
18	2.257	0.956	1.301	1.36	M	4.78
19	2.228	0.956	1.272	1.33	M	4.71
20	2.213	0.882	1.331	1.51	SM	4.68
21	2.176	0.934	1.243	1.33	M	4.60
22	2.110	0.875	1.235	1.41	M	4.46
23	2.088	0.904	1.184	1.31	M	4.42
24	1.993	0.934	1.059	1.13	M	4.22
25	1.941	0.860	1.081	1.26	M	4.11
26	1.890	0.860	1.029	1.20	M	4.00
27	1.860	0.750	1.110	1.48	M	3.94
28	1.860	0.794	1.066	1.34	M	3.94
29	1.676	0.699	0.978	1.40	M	3.55

*Class: M - metacentric; SM - submetacentric.

Fernández et al. (2000) reported differing karyogram results for yacon, noting 11 submetacentric pairs, two subtelocentric pairs, and 16 metacentric pairs. They observed that yacon chromosomes range in size from 3.12 µm (chromosome 1) to 1.80 µm (chromosome 29), with a satellite pair on the short arm of chromosome 15, leading to a karyotypic formula of 32 M + 22 SM + 4 A (acrocentric).

This classical cytogenetic characterization of yacon has laid the foundation for understanding potential karyotypic alterations in the species. The data generated provide essential qualitative and quantitative information, promoting further research into yacon cytotaxonomy.

The observed differences in chromosome numbers and genetic material across studies may stem from several factors. Environmental conditions like temperature, altitude, and soil composition can induce genomic changes and chromosomal rearrangements. Variations in cytogenetic techniques and the resolution of chromosome counting and karyotyping may also contribute to discrepancies. Additionally, genetic diversity within yacon populations, potentially due to hybridization and polyploidy, could lead to diverse karyotypic patterns. Future research should aim to conduct detailed comparative analyses using standardized cytogenetic methods across various yacon populations and environmental settings.

Conclusion

The mean nuclear DNA 2C content of yacon is 6.390 ± 0.0017 pg, with a 1C value of 3.195, which is equivalent to 3,124.710 Mbp. This 1C value classifies the S. yacon genome as small. The observed 1C and 2C nuclear values and chromosome count of 2n = 58 suggest that various yacon germplasms have been adapted to tropical climates. This study lays a foundational understanding of the genetic and cytogenetic characteristics of yacon cultivated outside its native Andean region. The small genome size may confer adaptability and stress tolerance advantages, making these findings pivotal for breeding programs aimed at improving yacon's agricultural performance, particularly in tropical environments. Further research should focus on expanding genetic studies to explore yacon population diversity across different regions, aiming to identify genetic markers linked to desirable traits such as disease resistance and yield enhancement. Incorporating these genetic insights into breeding strategies will help develop yacon cultivars tailored to specific environmental conditions and market needs. By pursuing these research avenues, we can enhance our understanding and cultivation of yacon, bolstering its global adoption as a functional food crop.

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