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Chemical profile analysis of germinated *in vitro* seedlings of *Diospyros anisandra*

Análisis del perfil químico de plántulas germinadas *in vitro* de *Diospyros anisandra*

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ABSTRACT

Diospyros anisandra is a tree known for its valuable metabolites; however, its application to commercial production has been limited. *In vitro* culture is a viable option to obtain their metabolites for this purpose. This study revealed a significant advance by achieving, for the first time, the successful establishment of *in vitro* germination and phytochemical analysis of seedlings obtained in these controlled systems for *Diospyros anisandra.* We explored a method of scarification and stratification to accelerate the germination. It was found that adding gibberellic acid (GA3) to Murashige and Skoog's (MS) nutrient medium led to 73% germination at a concentration of 57.8 µM GA3. Afterward, in whole seedlings, bioactive metabolites such as plumbagin, stigmasterol, β-sitosterol, taraxasterol, vitamin E, and betulonic acid were detected in the seedlings 90 days after planting. These are very similar to what plants have in their natural state. Therefore, *in vitro* cultivation emerged as a strategy that allows for the easy obtaining of plant material, thereby favoring the extraction of compounds in relatively short periods. Overall, this study lays a solid foundation for exploring the biotechnological potential of *D. anisandra.*

Keywords: in vitro, chemical profile, bioactive metabolites, biotechnological.

RESUMEN

Diospyros anisandra es un árbol conocido por sus valiosos metabolitos; sin embargo, su aplicación a la producción comercial ha sido limitada. El cultivo *in vitro* es una opción viable para obtener sus metabolitos con este fin. Este estudio revela un avance significativo al lograr, por primera vez, el establecimiento exitoso de la germinación *in vitro* y el análisis fitoquímico de plántulas obtenidas en estos sistemas controlados para *Diospyros anisandra*. Exploramos un método de escarificación y estratificación para acelerar la germinación. Se encontró que la adición de ácido giberélico (GA3) al medio nutritivo de Murashige y Skoog (MS) condujo a una germinación del 73% a una concentración de 57.8 µM de GA3. Posteriormente, en plántulas enteras, se detectaron metabolitos bioactivos como plumbagina, estigmasterol, β-sitosterol, taraxasterol, vitamina E y ácido betulónico en las plántulas 90 días después de la siembra. Estos son muy similares a los que las plantas tienen en su estado natural. Por lo tanto, el cultivo *in vitro* surge como una estrategia que facilita la obtención de material vegetal, favoreciendo así la extracción de compuestos en periodos relativamente cortos. En general, este estudio sienta una base sólida para explorar el potencial biotecnológico de *D. anisandra.*

Palabras claves: in vitro, perfil químico, metabolitos bioactivos, biotecnológico.

INTRODUCTION

Obtaining a significant amount of bioactive compounds from medicinal plants is difficult, especially in the case of *Diospyros anisandra S.F. Blake*. This particular species contains valuable metabolites that have been found to have significant properties such as anti-tuberculous, antibacterial, larvicidal, antioxidant, and recently antiviral effects (Cetina-Montejo et al., 2019; Flota-Burgos et al., 2020; Juárez-Méndez et al., 2022; Rosado-Aguilar et al., 2010; Uc-Cachón et al., 2014). The slow growth of this species shows challenges for traditional cultivation, hindering the effective production of biotechnologically significant compounds. Furthermore, environmental factors have a significant impact on the accumulation of metabolites within the plant, which will also affect the plant's state of development and growth (Reshi et al., 2023). In this situation, biotechnology appears to be a viable solution to these pro-

blems. It simplifies the process of obtaining desired metabolites in a more regulated and effective manner. This can be achieved by utilizing techniques such as *in vitro* plant regeneration, crop optimization, and genetic engineering to produce more secondary metabolites (Karuppusamy, 2009). Different studies reported the establishment of *in vitro* culture for the *Diospyros* genus. The effect of gibberellic acid, auxins, and cytokinins on the germination of *Diospyros hispida* was assessed, revealing negative effects (Ramalho & Braga, 2021) In contrast, Khan (2016) reported for *Diospyros ebenum* pre-sowing seed treatments, such as different concentrations of H_2SO_4, KCl, hot water, and distilled water, influence the germination percentage. However, there is no previous research regarding establishing an *in vitro* culture in *D. anisandra*. This lack of studies represents a significant research opportunity to explore propagation methods that could present a viable alternative. This strategy is designed to contribute to the general advancement of phytochemical research in this species. Therefore, our main objective is to identify the chemical profiles of seedlings obtained *in vitro*, providing detailed information about their bioactive compounds. Given the ongoing growth in demand for natural products in the international market, the production of secondary metabolites through *in vitro* methods has become a relevant alternative. It allows production to be independent of biotic and abiotic environmental factors and reduces production costs by establishing rigorous quality control systems and simplifying extraction processes (Mulabagal & Tsay, 2004; Paek et al., 2005). This study tries to fill in a gap in knowledge by giving important details that will help us learn more about *D. anisandra* and develop better ways to make secondary biomolecules efficiently and sustainably.

MATERIALS AND METHODS

Collection of biological material. The plant germplasm was collected in the botanical Garden of the Yucatan Scientific Research Center. Green fruits were selected, which matured in cardboard boxes, under dark conditions at 25 °C ± for seven days. 100 seeds were obtained from fifty fruits collected. The morphology of the fruits was previously evaluated according to the protocol described by Hamalton et al. (2020).

Seed stratification, scarification, and disinfection. For the sterilization process, four batches of 10 seeds were used, with three replicates each. Seed preparation included removing the pulp by rinsing with water and Tween-80 for 10 minutes, under continuous agitation, followed by three rinses with distilled water. The seeds were then placed on paper for one hour to dry. In a sterile laminar flow hood, the seeds were immersed in 70% (v/v) ethyl alcohol for 5 minutes. Subsequently, they were disinfected in a solution of sterile water with commercial sodium hypochlorite at concentrations of 20%, 25%, and 30% with 1 mL of antifungal agent per mL of solution (v/v) for 20, 25, and 30 minutes, respectively. Finally, the seeds were rinsed three times with sterile distilled water and sown in the corresponding culture medium, as described by Sanchez et al. (2002). On the other hand, stratification was carried out by exposing 25 seeds to 4 °C in distilled water, in total darkness, for 15 days, with three replicates. Simultaneously, another 25 seeds were left untreated, as a control group. Regarding scarification, two different methods were used: the seed coats were sanded with paper sandpaper (no. 120), and then subjected to a process of disinfection with sodium hypochlorite (30%). In the second option, after disinfection with sodium hypochlorite (30%), a cut was made in the upper part of the seed coat of 25 seeds using sterilized tweezers and a scalpel, all carried out under aseptic conditions, each treatment being performed in triplicate. Finally, 25 untreated seeds were maintained as a control group. The experimental design was established according to Pie and Schreiber (2016)

Mediums and culture conditions. All experiments were performed using MS medium, with 4.33 g L^{-1} of Murashige & Skoog PhytoTech inorganic salts added, along with 10 mL of a stock solution of MS vitamins (thiamine, myo-inositol, pyridoxine, and glycine) and 30 g L^{-1} of sucrose. The pH was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl, and finally, 8 g L^{-1} of CAISSON micropropagation agar was added. Culture media were distributed in glass flasks with 20 mL of medium and autoclaved at 121 °C and 1 kg cm^{-2} for 15 minutes, then stored at room temperature for later use.

Samples preparation and extraction. Whole *D. anisandra* seedlings were powdered in a mortar with liquid nitrogen; One gram of plant material was dissolved with hexane, and in Branson Ultrasonics equipment, it was sonicated for 30 minutes. Subsequently, the sample was centrifuged, and the supernatant was evaporated for 24 hours. This process was carried out in triplicate.

GC-MS and TLC Analysis. Each extracted sample was dissolved in chloroform (1 mg mL^{-1}) and subjected to analysis using an Agilent Technologies 6890-N gas chromatograph coupled to an Agilent Technologies 5975B inert mass selective detector (MDS). Chromatographic fractionation was repeated three times to ensure the reproducibility of the experiment. Analysis was performed on an Ultra 1 capillary column (25 m x 0.321 mm i.d.; 0.52 µm d.f.; 100% dimethylpolysiloxane). Helium was used as a carrier gas at a constant pressure of 1.5 mL min^{-1}. The initial oven temperature was maintained at 180 °C for 5 minutes and then increased to 300 °C at 10 °C min^{-1}.

The compounds present in each extract were subjected to spectroscopic quantification. This was achieved utilizing a Hewlett-Packard 5,890 gas chromatograph outfitted with an Ultra 1 capillary column (dimethylpolysiloxane,

100%, 25 m × 0.321 mm i.d.; 0.52 μm df). As a carrier gas, nitrogen was utilized at a constant pressure of 1 mL min^{-1}. After maintaining the initial oven temperature at 180 °C for 5 minutes, it was progressively raised to 300 °C for 20 minutes at a rate of 10 °C min^{-1}. The described methodology was reported by Uc-Cachón et al. (2013). calibration solutions were prepared in chloroform, in a range of concentrations between 0.1 and 7 μg μL^{-1}.

Thin layer chromatography (TLC) was carried out using Merck brand silica gel 60 F254 coated plates. A mix of hexane and ethyl acetate (8:2) was used as the mobile phase. The extracts and standards were applied to the bottom of the plate and the mobile phase rose by capillarity. After development, the application points were visualized and compared with purified standards to identify the compounds present in the extracts.

Data collection, and statistical analysis. For the colorimetry study, Image-J software was used to obtain the values of the number of pixels present in each color of the different images. Statistical analysis was carried out using Statgraphics software to evaluate the distribution and proportion of the values obtained in each color channel in the various images, using the methodological approach described by Apriantoro et al. (2018).

On the other hand, to determine the germination rate was calculated by dividing the number of germinated seeds by the total number of seeds and multiplying the result by 100, as reported by Ramalho and Braga (2021). Finally, the R Studio software, together with ComplexHeatmap, dendextend and circlize libraries were used to create the heat map that represents the chemical profile of the metabolites identified by gas chromatography coupled to mass spectrometry (GC/MS).

RESULTS

Morphological characterization of fruit and seed maturation in D. anisandra. The morphological characteristics of the fruits and seeds were studied during the maturation process. The immature fruits, which were spherical and green in color and had a diameter of 2 cm, changed to a reddish tone after seven days, signaling their ripening and facilitating the collection of seeds. However, it was observed that when the fruits acquired a dark color, the seeds were moldy and adhered to the pulp, making them difficult to extract. The colorimetric analysis results revealed significant changes in the chromatic characteristics of the fruits throughout the maturation process. A trend was observed towards an increase in luminosity and saturation as the fruits reached full maturity. In addition, variations in tone were found, with a gradual transition from greener and yellow tones to more reddish tones in mature fruits (Figure 1A). The color values obtained were consistent, with a P value of 0.0007 and a confidence level of 95.0% (Supplementary Table 2 and Supplementary Figure 1). On the other hand, mature seeds are brown and oval, with a length and diameter that vary between 7 mm to 10 mm and 3 mm to 5 mm, respectively (Figure 1B). These findings highlight the importance of harvesting fruits at the right time to ensure seed quality.

Decontamination, scarification, stratification, and seed germination. For the decontamination of seeds, it was observed that the concentration of 30% sodium hypochlorite for 30 minutes achieved a contamination index of 43%, while compared with other treatments, it was observed that after six days of sowing, most of the seed culture was colonized by fungi up to 63%. It was added 1 μl mL^{-1} of antibiotic and antifungal stock solution (Caisson 100x brand) on the MS medium, to counteract this, which resulted in 5% contamination (Supplementary Figure 1).

On the other hand, seeds subjected to stratification showed a significant increase in the germination percentage compared to non-stratified seeds (Table 1). The scarification treatment, scalpel cutting the seeds after sterilization (Figure 1C), resulted in 73% germination (Table 1), while mechanical friction of the outer shell before decontamination resulted in germination of less than 17% (Table 1). Regarding the GA3 treatments, the concentration of 57.8 μM promoted optimal growth; After 5 days, the radicle exit was evident (Figure 1D). After 20 days, continued plant growth was noted, although the outer covering had not yet shed (Figure 1E). At 40 days, a more pronounced development was observed compared to previous stages; many plants had true leaves (Figure 1F). Finally, after 60 days, a greater number of leaves were observed, with an approximate height of 5 cm and a root length of 6 cm, thus completing the growth cycle observed in the study (Figure 1G). In contrast to the GA3 treatment, there were clear differences in growth in standard MS medium and sterile water, with few seeds germinating and radicles that were no longer than 2 cm long (Figure 2).

Chemical profile of in vitro seedlings and wild plants. To identify secondary metabolites from seedlings grown *in vitro*, various compounds were detected and visualized on a silica plate using phosphomolybdic acid and UV light. In this way, secondary metabolites were identified, such as vitamin E, stigmasterol, β-sitosterol, lupeol, betulin, betulinic acid, and plumbagin. To confirm the results were accurate and reliable, the retention factors (Rf) of pure standards were compared with those of seedling extracts grown in a lab (Supplementary Figure 2).

On the other hand, gas chromatography coupled with mass spectrometry made it possible to corroborate the molecular characteristics of the compounds, such as plumbagin, vitamin E, stigmasterol, β-sitosterol, taraxasterol, and betulin. This identification was carried out by comparing the retention times and mass fragmentation with the National Institute of Standards and Technology (NIST) database, in addition to injecting pure standards

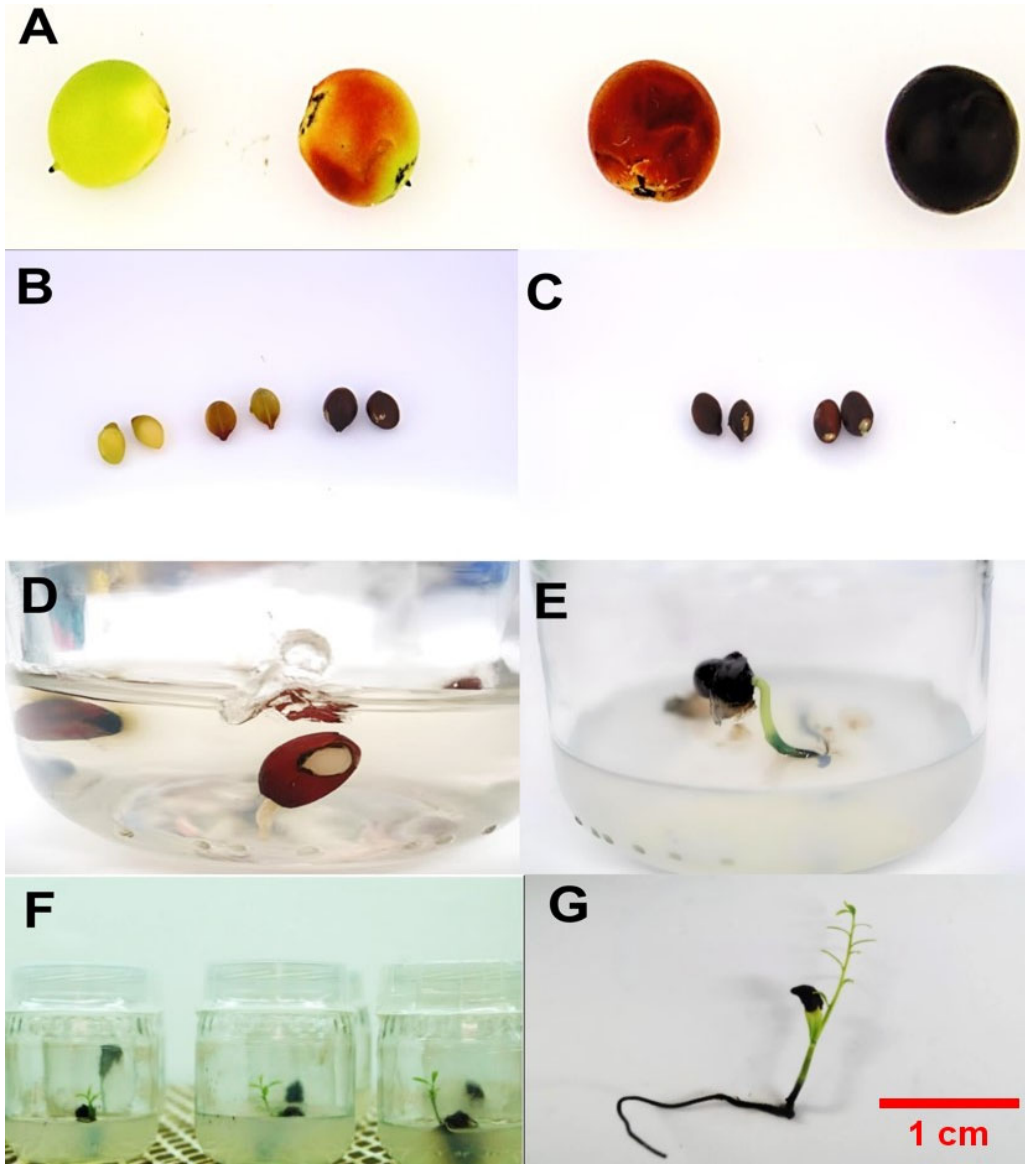

Figure 1. *In vitro* seedling growth in controlled environments. A) Fruit ripening stages. B) Color difference between mature and immature seeds. C) Unscarified and scarified seed treatment with a scalpel. D) Radicle emergence after 5 days of the *in vitro* plant growth. E) Plant grown after 20 days, with the outer cover still in place. F) Plant grown after 40 days, a more pronounced development was observed compared to previous stages, most plants presented true leaves with a more advanced development. G) Seedlings grown.

Crecimiento de plántulas *in vitro* en ambientes controlados. A) Etapas de maduración del fruto. B) Diferencia de color entre semillas maduras e inmaduras. C) Tratamiento de semillas escarificadas y no escarificadas con bisturí. D) Emergencia de la radícula a los 5 días de crecimiento de la planta *in vitro*. E) Planta cultivada a los 20 días, con la cubierta externa aún en su lugar. F) Planta cultivada a los 40 días; se observó un desarrollo más pronunciado en comparación con etapas anteriores; la mayoría de las plantas presentaron hojas verdaderas con un desarrollo más avanzado. G) Plántulas cultivadas.

(Table 2) (Juárez-Méndez et al., 2022; Uc-Cachón et al., 2013, 2014). On the other hand, the gas-mass chromatogram of the seedlings *in vitro* exhibits peaks of similar compounds to the plants grown in a greenhouse (Figure 3). Both the height and area of the peaks provided information about the relative abundance of each component. Taking the above into account, the metabolites present in each sample, replicated in triplicate, were explored through heatmap analysis. In this analysis, the horizontal and vertical axis represent the information of the sample and the variable, respectively. Red color indicates a high relative concentration, while green color indicates a low relative concentration. Figure 4, shows that stigmasterol, beta-sitosterol, plumbagin and palmitic acid are the most abundant compounds compared to samples of plants grown in the greenhouse.

Table 1. Germination results for scarification and stratification treatments. Significant differences are observed in the percentage of treatments, as detailed in Supplementary Tables 2 and 3, where the statistical differences between each treatment are indicated.

Resultados de germinación de los tratamientos de escarificación y estratificación. Se observan diferencias significativas en el porcentaje de tratamientos, como se detalla en las Tablas Suplementarias 2 y 3, donde se indican las diferencias estadísticas entre cada tratamiento.

Seed	N° stratification	Stratification	Number seeds	Germination days	Number plants	Germination percentage	Culture medium
Without scarifying	X		30	60	0	0	57.8 µM gibberellic acid
Outer layer wear	X		30	60	3	10	
cross-section cut	X		30	60	5	17	
Without scarifying		X	30	60	0	0	
Outer layer wear		X	30	60	6	20	
Cross-section cut		X	30	60	22	73	

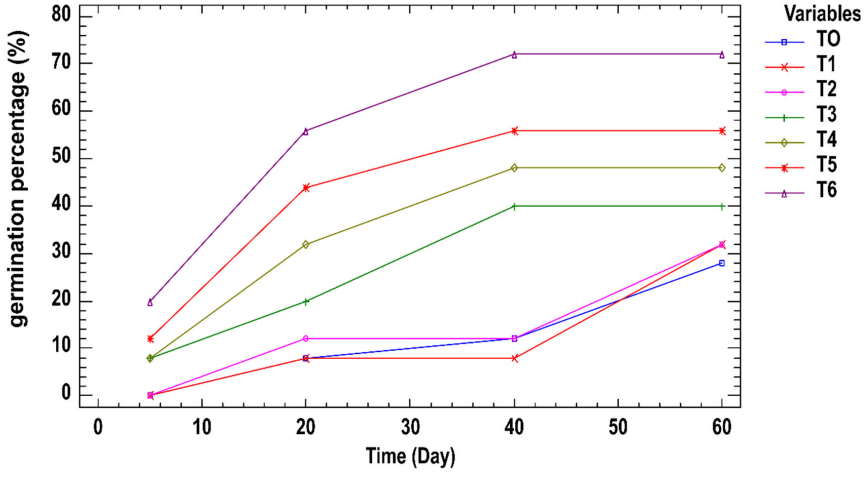

Figure 2. Seed germination response using different growth media. The micronutrient and macronutrient composition of each growth medium, T0, T1, T2, T3, T4, and T5, is described in Supplementary Table 1. Similarly. Supplementary Table 2 shows the differences in the statistical analysis.

Respuesta de germinación de semillas utilizando diferentes medios de cultivo. La composición de micronutrientes y macronutrientes de cada medio de cultivo (T0, T1, T2, T3, T4 y T5) se describe en la Tabla Suplementaria 1. De igual forma, la Tabla Suplementaria 2 muestra las diferencias en el análisis estadístico.

DISCUSSION

The methodology used in this study represents a considerable advance in *D. anisandra* research since it allows for the analysis of the chemical profile of seedlings obtained through *in vitro* germination. This approach provides a detailed view of the bioactive compounds present under controlled growth conditions, an aspect little explored until now in *D. anisandra* seedlings. Therefore, *in vitro* germination of *D. anisandra* seeds emerges as a highly efficient technique to obtain high-quality seedlings. According to our study, a concentration of 20 mg L^{-1} of GA3 achieved uniform germination in a short period, resulting in a germination rate of 73%. Previous research by Hamalton et al. (2020) and Jeyavanan et al. (2016), found that *in vitro* germination is an effective strategy for propagating the *Diospyros* genus. Additionally, the results are similar to those found by Ramalho and Braga (2021), who showed that using gibberellic acid (GA3) greatly enhances seedling growth and germination in *Diospyros hispidia*. Similarly, the effectiveness of scarification and stratification methods has also thisbeen confirmed in previous studies by Sánchez-Soto et al. (2017). These results underline the feasibility and effectiveness of *in vitro* germination of *D. anisandra* seeds as a promising strategy to obtain high-quality seedlings in a short period.

In the case of the phytochemical profiles of *D. anisandra* seedlings, they have identified a diversity of bioactive compounds with anti-inflammatory, antitumor, antimicrobial, and, lately, antiviral properties. Recent studies have characterized new compounds with potential for biological activity (Flota-Burgos et al., 2020; Ortiz-López et al., 2022;

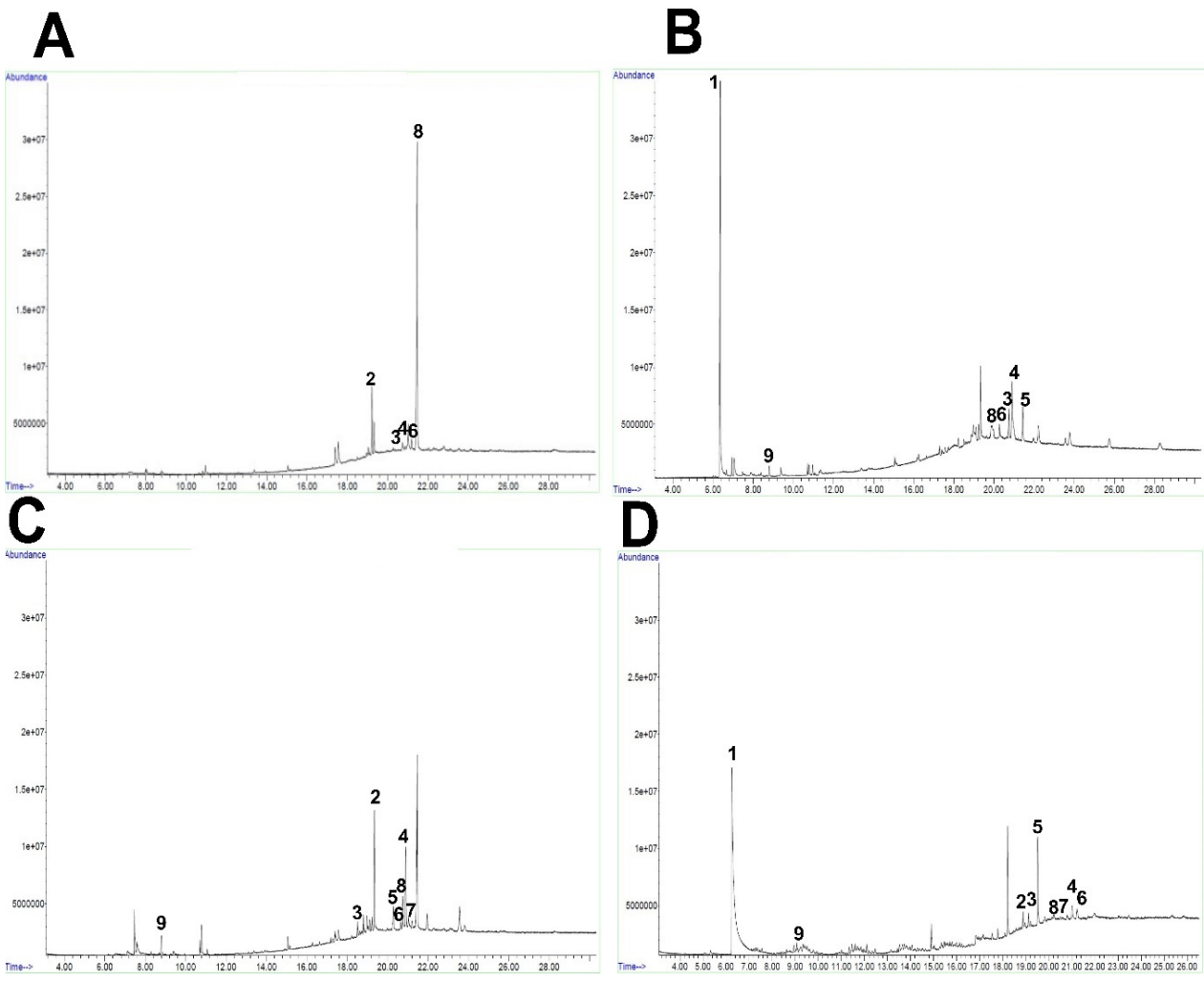

Figure 3. Chromatographic profiles of the volatile compounds obtained by GC-MS. (A) chromatogram of leaf of a greenhouse plant. (B) Chromatogram of the stem bark of a greenhouse plant. (C) Chromatogram of roots of greenhouse plants. (D) Chromatogram of whole seedlings grown *in vitro*. Each sample allows us to identify differences in the composition of volatile compounds between greenhouse plants and those grown *in vitro*. Information on the data of secondary metabolites found in seedlings grown *in vitro* and in greenhouse plants, according to their retention time, was also reported in Table 2.

Perfiles cromatográficos de los compuestos volátiles obtenidos por GC-MS. (A) Cromatograma de la hoja de una planta de invernadero. (B) Cromatograma de la corteza del tallo de una planta de invernadero. (C) Cromatograma de las raíces de plantas de invernadero. (D) Cromatograma de plántulas completas cultivadas *in vitro*. Cada muestra permite identificar diferencias en la composición de compuestos volátiles entre las plantas de invernadero y las cultivadas *in vitro*. La Tabla 2 también *presenta información sobre los metabolitos secundarios encontrados en plántulas cultivadas in vitro* y en plantas de invernadero, según su tiempo de retención.

Uc-Cachón et al., 2014). These findings suggest that *D. anisandra* seedlings could be a valuable source of natural compounds with pharmacological applications. Furthermore, researchers recognize the potential of *in vitro* culture techniques to produce secondary metabolites, as discussed in the work of Karuppusamy (2009). However, further research is necessary to fully comprehend the biological activities of these compounds and their potential for medicinal use

Our results could have serious consequences for various industries, including pharmaceutical, food, agricultural, and cosmetics, by suggesting new possibilities for the production and application of bioactive compounds derived from *D. anisandra*. For example, vitamin E is effective in protecting against oxidative damage in cells, making it an essential ingredient in both pharmaceuticals and cosmetics (Rizvi et al., 2014). Furthermore, the combination of beta-sitosterol, stigmasterol, and campesterol, present in vegetable margarines, is not only widely used in the food industry, but also has important pharmaceutical implications. These phytosterols have been shown to reduce LDL cholesterol levels in individuals with hypercholesterolemia, highlighting their potential to address cardiovascular health issues (Poli et al., 2021).

Table 2. Data on secondary metabolites obtained and found in different explants of greenhouse plants, and whole seedlings grown *in vitro*, including their retention time (Rt), comparison with pure standard (STD), comparison with mass fragmentation (MF) and thin layer chromatography (TLC).

Datos sobre metabolitos secundarios obtenidos y encontrados en diferentes explantos de plantas de invernadero y plántulas enteras *cultivadas in vitro*, incluyendo su tiempo de retención (Rt), comparación con estándar puro (STD), comparación con fragmentación de masa (MF) y cromatografía en capa fina (TLC).

Plant explants	peak number	Compound	retention time	Similarity (%)	STD*	MF*	TLC*
Whole *in vitro* plant	1	Plumbagin	06.26	99	x	x	x
	2	Vitamin E	19.45	99	x	x	x
	3	Stigmasterol	19.16	99	x	x	x
	4	Taraxasterol	21.20	90		x	x
	5	β-sitosterol	20.45	95	x	x	x
	6	Lupeol	21.20	90	x	x	x
	7	Betulin	20.90	95	x	x	x
	8	α-amiryn	20.80	95	x	x	x
	9	Acid palmitic	09.20	97		x	
Greenhouse leaf	2	Vitamin E	19.16	99	x	x	x
	3	Stigmasterol	20.54	99	x	x	x
	4	Taraxasterol	21.20	74		x	x
	8	α-amiryn	21.26	74	x	x	x
	6	Lupeol	20.95	95	x	x	x
	5	β-sitosterol	20.53	90	x	x	x
	7	Betulin	20.90	95	x	x	x
Greenhouse stem bark	2	Vitamin E	19.16	99	x	x	x
	3	Stigmasterol	21.66	99	x	x	x
	4	Taraxasterol	21.17	74		x	x
	5	β-sitosterol	20.52	99	x	x	x
	1	Plumbagin	06.20	94	x	x	x
	6	Lupeol	21.20	90	x	x	x
	8	α-amiryn	21.80	95	x	x	x
	7	Betulin	20.90	95	x	x	x
	9	Acid palmitic	08.90	97		x	
Greenhouse root	2	Vitamin E	19.16	98	x	x	x
	8	α-amiryn	20.85	90	x	x	x
	5	β-sitosterol	20.53	90	x	x	x
	4	Taraxasterol	21.83	77		x	x
	3	Stigmasterol	18.93	99	x	x	x
	6	Lupeol	20.90	90	x	x	x
	7	Betulin	21.20	95	x	x	x
	9	Acid palmitic	08.64	97		x	

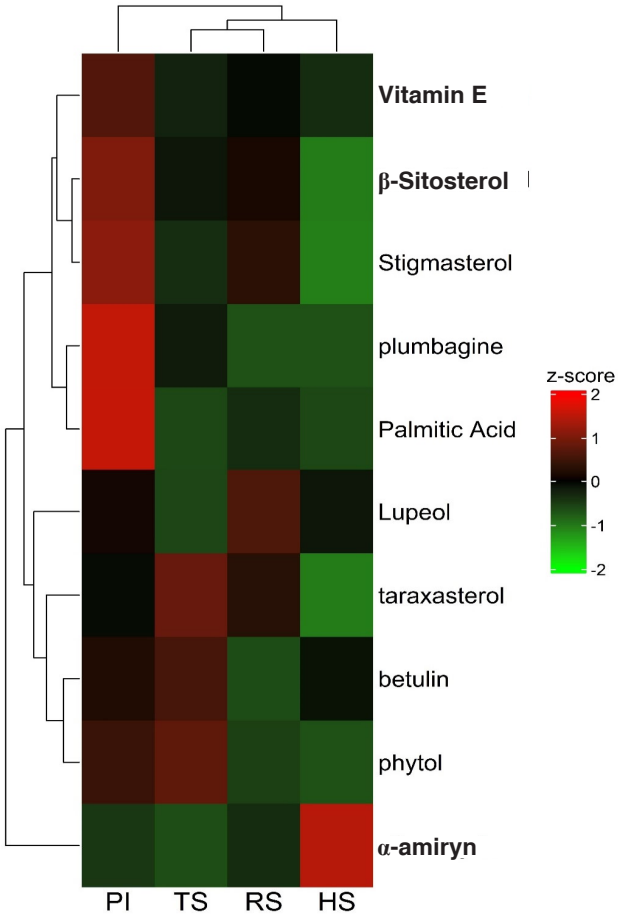

Figure 4. Heat map illustrating the concentration of metabolites in different samples. The colors represent the abundance of metabolites, with red indicating a high relative concentration, while green reflects a low relative concentration. The samples analyzed include *in vitro* seedlings (PI), the stem of a greenhouse plant (TS), the root of a greenhouse plant (RS), and the leaf of a greenhouse plant. Three replicates of each sample were analyzed.

Mapa de calor que ilustra la concentración de metabolitos en diferentes muestras. Los colores representan la abundancia de metabolitos: el rojo indica una concentración relativa alta y el verde una concentración relativa baja. Las muestras analizadas incluyen plántulas *in vitro* (PI), tallos de plantas de invernadero (TS), raíces de plantas de invernadero (RS) y hojas de plantas de invernadero. Se analizaron tres réplicas de cada muestra.

It is important to highlight that those seedlings obtained *in vitro* constitute a continuous source of plant material for phytochemical analyses and represent a valuable resource for the genetic manipulation of secondary metabolism. This capacity opens new perspectives for the controlled production of bioactive compounds of commercial interest. It suggests significant potential for the study of the biosynthesis pathways of the compounds found under controlled conditions, as well as the development of more effective and sustainable strategies for obtaining *D. anisandra* metabolites.

CONCLUSION

This study provides an important milestone in the characterization of secondary metabolites of *D. anisandra*, especially in seedlings grown *in vitro*. The germination of *D. anisandra* seeds is highly efficient, achieving a notable germination rate of 73%, surpassing previous studies. The phytochemical analysis identifies compounds with known therapeutic properties, pointing to promising potential for pharmaceutical, food, and cosmetic applications. Furthermore, it offers a reliable source of material for *in vitro* analysis, bioactive compound production, and genetic modification. This approach opens new avenues for the sustainable obtaining of metabolites from *D. anisandra*, with important commercial and therapeutic applications.

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AUTHORS CONTRIBUTION

All authors contributed to the conception and design of the study. Material preparation, data collection, and laboratory analysis were performed by A. A. Experimental design and chromatographic data analysis were performed by F. E. Corrections to the draft of the manuscript were performed by M. A. H., R. B., L. C. R. and E. C. All authors commented on earlier versions of the manuscript. All authors read and approved the final manuscript.

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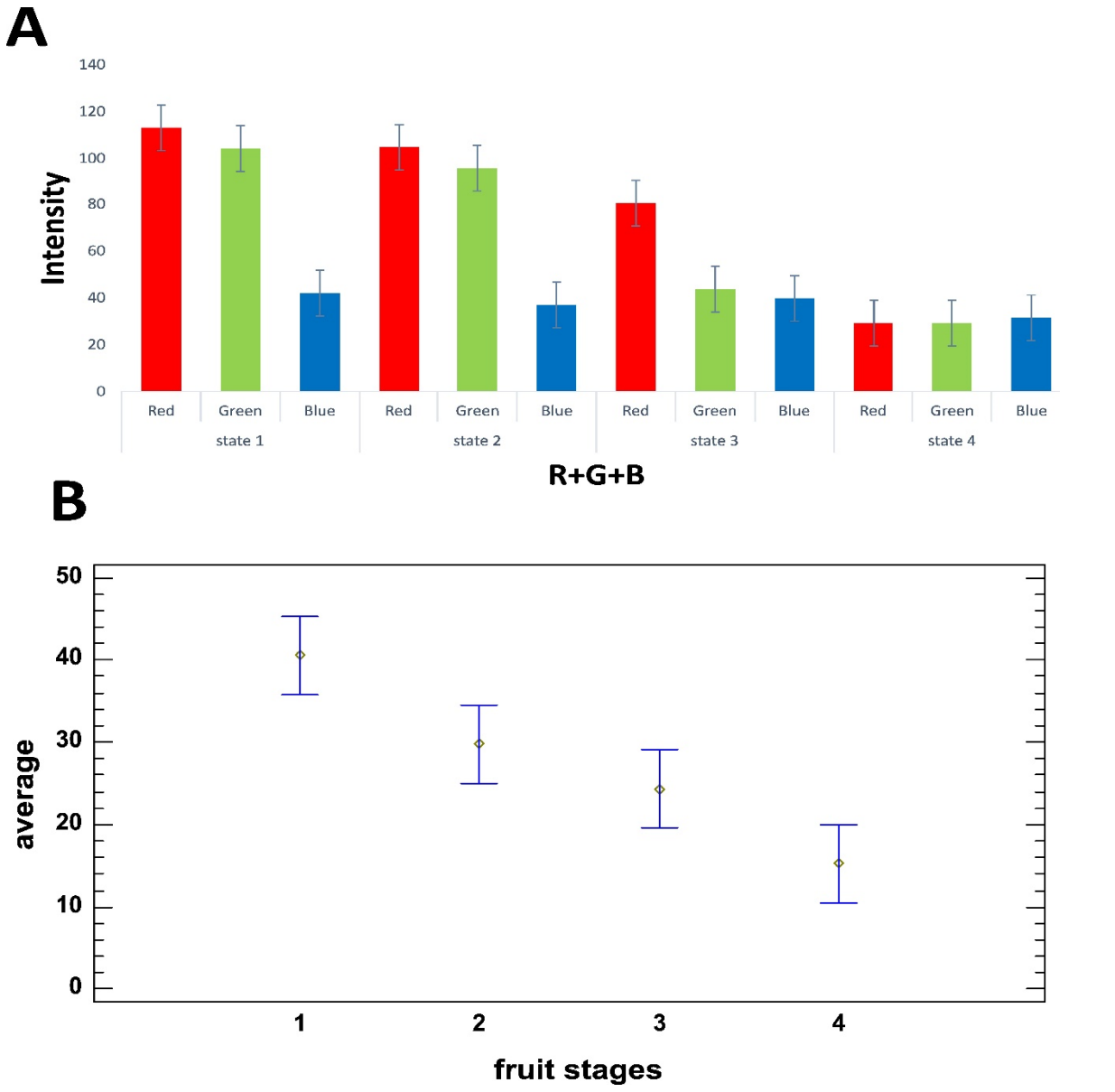
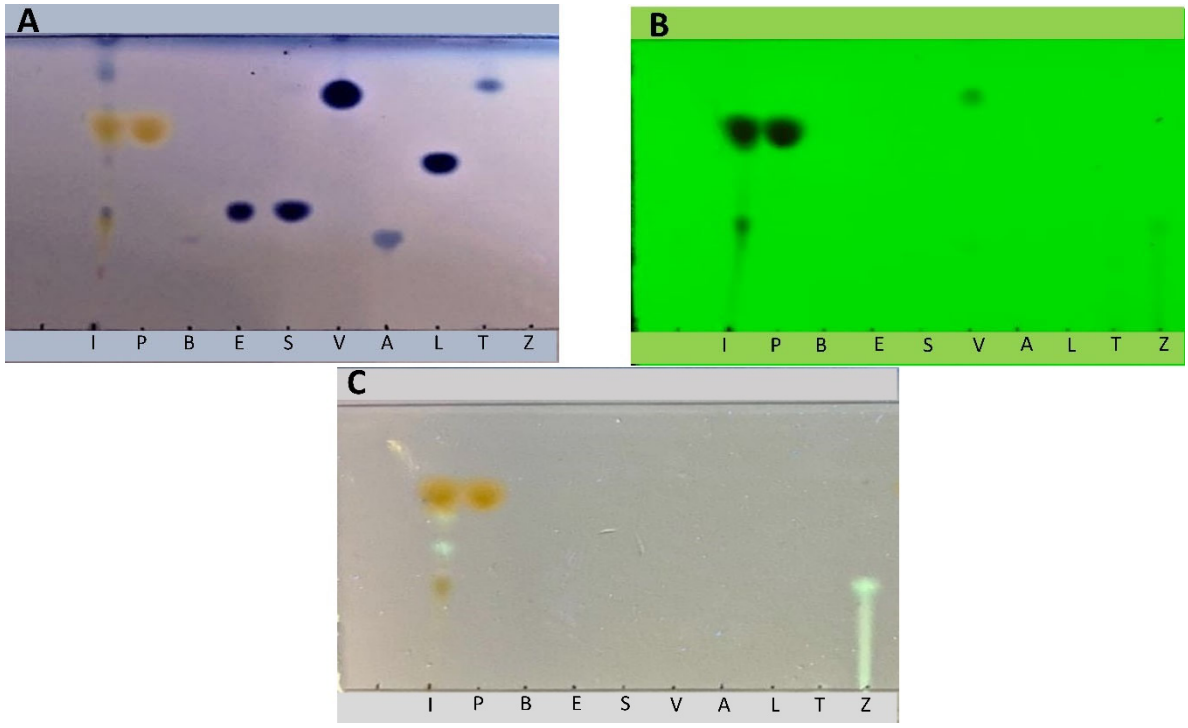
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Evaluation of fruit color for seed maturation. A) The bar graph shows red, green, and blue (RGB) values related to the area of the fruit; data was obtained with Image-J software. B) Graph of means and standard error shows a great difference in the mean of each sample, which points to a link between the color of the fruit and the maturity of the seed. Three replicates of each sample were analyzed.

Evaluación del color del fruto para la maduración de la semilla. A) El gráfico de barras muestra los valores de rojo, verde y azul (RGB) relacionados con el área del fruto; los datos se obtuvieron con el software Image-J. B) El gráfico de medias y error estándar muestra una gran diferencia en la media de cada muestra, lo que indica una relación entre el color del fruto y la madurez de la semilla. Se analizaron tres réplicas de cada muestra.



Supplementary Figure 2. Comparison between pure standards and *in vitro* plant extract on silica plate. A) Plate developed with phosphomolybdic acid. B) Plate developed with 260 nm shortwave UV light. C) Plate developed with 360 nm long-wave UV light. The following reference compounds were identified on the plate by letters: *in vitro* plant (I), Plumbagin (P), Betulin (B), Stigmasterol (E), β-sitosterol (S), Vitamin E (V), Betulinic Acid (A), Lupeol (L), Taraxasterol (T) and zeylanone epoxide (Z).

Comparación entre estándares puros y extracto vegetal *in vitro* en placa de sílice. A) Placa revelada con ácido fosfomolíbdico. B) Placa revelada con luz UV de onda corta de 260 nm. C) Placa revelada con luz UV de onda larga de 360 nm. Los siguientes compuestos de referencia se identificaron en la placa con letras: Planta *in vitro* (I), Plumbagina (P), Betulina (B), Estigmasterol (E), β-sitosterol (S), Vitamina E (V), Ácido Betulínico (A), Lupeol (L), Taraxasterol (T) y Epóxido de Zeilanona (Z).

Supplementary Table 1. Various treatments were applied to induce the germination of *D. anisandra* seeds, including stratification, scarification, and gibberellic acid. Each of these methods was applied in a controlled manner, and germination rates were recorded to evaluate their effectiveness.

Se aplicaron diversos tratamientos para inducir la germinación de las semillas de *D. anisandra*, incluyendo estratificación, escarificación y ácido giberélico. Cada uno de estos métodos se aplicó de forma controlada y se registraron las tasas de germinación para evaluar su eficacia.

Name of treatment	Number of seeds	Culture medium
T0	25	Sterile Water
T1	25	MS basal
T2	25	MS 1/2
T3	25	MS basal + 2.4 mg L^{-1} GA3
T4	25	MS basal + 5 mg L^{-1} GA3
T5	25	MS basal + 10 mg L^{-1} GA3
T6	25	MS basal + 20 mg L^{-1} GA3

Supplementary Table 2. The ANOVA table decomposes the color variance into two components: a between-groups component and a within-groups component. The F-ratio, which is 11.6776, is the quotient between the estimate between groups and the estimate within groups. Since the P value of the F-test is less than 0.05, there is a statistically significant difference between the mean of each fruit between one color level and another, with a 95.0% confidence level.

La tabla ANOVA descompone la varianza de color en dos componentes: un componente intergrupos y un componente intragrupos. El F-ratio, que es 11,6776, es el cociente entre la estimación intergrupos y la estimación intragrupos. Dado que el valor p de la prueba F es menor que 0,05, existe una diferencia estadísticamente significativa entre la media de cada fruta entre un nivel de color y otro, con un nivel de confianza del 95,0%.

Source	Sum of Squares	Gl	Square Medio	F-ratio	P value
Entre grupos	1,341.450	3	447.1490	11.68	0.0007
Intra grupos	459.494	12	38.2912		
Total (Corr.)	1,800.940	15			

Supplementary Table 3. The ANOVA table decomposes the variance of germination into two components: between groups and within groups. The F-ratio, 4.50528 in this case, is the ratio between the between-group component and the within-group component. With a P-value of the F-test less than 0.05, a statistically significant difference is established between the germination means between treatment levels, with a confidence level of 95.0%.

La tabla ANOVA descompone la varianza de la germinación en dos componentes: intergrupos e intragrupos. El F-ratio, en este caso 4,50528, es el cociente entre el componente intergrupos y el componente intragrupos. Con un valor P de la prueba F inferior a 0,05, se establece una diferencia estadísticamente significativa entre las medias de germinación entre los niveles de tratamiento, con un nivel de confianza del 95,0%.

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
Entre grupos	7,795.43	6	1,299.240	4.51	0.0044
Intra grupos	6,056.00	21	288.381		
Total (Corr.)	13,851.40	27			