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SECRETARIA DE AGRICULTURA Y DESARROLLO RURAL

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EVALUATING TIME SERIES PREDICTION MODELS: EGG PRICES IN MEXICO

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ABSTRACT

Mexico is the world's largest consumer of eggs, producing 3.05 million Mg in 2021. The high variation in wholesale prices is a feature of the egg production system, which is important to producers and government institutions that need to forecast future prices for activity planning. As a result, it is necessary to propose tools that can reliably predict egg prices. The goal of this paper was to compare the performance of various statistical models by analyzing the time series of egg prices using the Akaike index and forecast error to determine which model best predicts the wholesale price of white eggs. The models evaluated were the autoregressive integrated moving average model (ARIMA), ARIMA with interventions, ARIMA with transfers, and regression with ARIMA errors. Two time series were used: the wholesale price of white eggs, constructed with data from the National System of Information and Market Integration (SNIIM) and the Agrifood and Fisheries Information Service (SIAP), and egg imports, calculated with data from the Economic Information System. The latter was used as an exogenous variable to explain the price of eggs. Both cover the period from January 2006 to December 2021. According to the Akaike index, the model with the best adjustment was ARIMA (0,1,1)(1,0,1)[12] with interventions. In the evaluation of forecast error, the best models were the regression models with ARIMA (1,1,0)(1,0,1)[12] and ARIMA (1,1,0)(1,0,1)[12] errors with transfer.

Keywords: ARIMA, regression with ARIMA errors, ARIMA with interventions, ARIMA with transfers.

INTRODUCTION

In Mexico, eggs are a basic product in the food basket and one of the cheapest protein sources available. The country is the world's largest egg consumer, with a per capita consumption of 24 kg. During 2021, Mexico ranked sixth as the world's largest egg producer, with a production volume of 3.05 million Mg. The state of Jalisco was the

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main producer, with 1.6 million Mg. The country is the seventh-largest importer of eggs for human consumption (SIAP, 2022).

One of the characteristics of the egg product system in Mexico is the high variability in wholesale egg prices across the country. For example, in September 2006, when the weighted average price in the country was \$9.72 MXN kg^{-1}, the state of Colima recorded prices of \$7.00 MXN kg^{-1}, while Querétaro reached \$13 MXN per kilogram, which represents a variance of \$2.39 MXN. In another case, in March 2020, the average wholesale price of white eggs in the country was \$34.91 MXN kg^{-1}; in the state of Guerrero, prices of \$28 MXN kg^{-1} were recorded, while in Veracruz they reached \$64.57 MXN kg^{-1}, with a variance of \$42.12 MXN for the month (SNIIM, 2022).

The high variation makes it difficult to predict egg prices, which has an impact on producers and government institutions that need to estimate forward prices in order to plan their activities. It would therefore be useful to have a methodology for price prediction with some degree of reliability. Statistical models are among the most effective and, therefore, the most widely used alternatives for time series forecasting. Among the most widely used are autoregressive (AR), moving average (MA), autoregressive with moving averages (ARMA), autoregressive integrated with moving averages (ARIMA), and the Holt Winter smoothing model (Brockwell and Davis, 2002).

ARIMA models have been used to forecasts in a variety of research areas. For example, Alburquerque and de Moraes (2007) forecast the average monthly cocoa price paid to producers in Brazil. The analysis of the variable with ARIMA models helps investors and producers form expectations about the future price behavior of this commodity. Sánchez-López *et al*. (2013) found a decrease in future bovine milk production in Baja California, Mexico, and recommended that government agencies generate forecasts that allow producers to take technical measures to deal with these circumstances, thereby improving their response capacity.

The diversity of existing models for time series forecasting generates a constant search to improve, evaluate, and propose new models with the aim of identifying the one with the best predictions for a given case. Box and Tiao (1975) improved the traditional ARIMA model by incorporating the effects of natural or induced interventions into the study variable, such as economic shocks, weather events, pandemics, and administrative changes, or by transferring the performance of another variable over time that may influence the direction of the time series. This is known as ARIMA models with interventions, ARIMA models with transfers, and regression with ARIMA errors, and their use can help improve the forecasting accuracy of the traditional ARIMA model.

Nowadays, productive, commercial, and service activities generate and store a large amount of information to analyze and use in decision-making. It can be generated at regular time intervals, either daily, weekly, monthly, or annually. Such information is commonly referred to as a time series, in which patterns of behavior can be identified and their short- to medium-term values predicted. Models for time series are used to obtain future values for a dataset, predictions supported by historical information on the series. In this sense, the models developed by Box and Jenkins (1973) have been used to make short-, medium-, and long-term predictions in stationary series, i.e., the mean, variance, and covariance are constant over time (Tsay, 2010). The stages of the Box-Jenkins methodology correspond to stationarity verification, differencing (if required), parameter identification and estimation, residual diagnosis, and prediction (Hanke and Wichern, 2010).

This method has been used extensively. For example, in Mexico, a study was conducted for ball tomato price forecasting with a time series from December 2008 to November 2009. The study used the Box-Jenkins methodology to identify an econometric ARIMA model, which has two autoregressive factors and one moving average (Marroquín-Martínez and Chalita-Tovar, 2011). In Venezuela, a statistical model was used to analyze inflation with a time series covering the period from June 1995 to July 2000; applying the Box-Jenkins methodology, the model that best fit the time series was the ARIMA with a confidence interval of 95 % (Seijas, 2002). Luis-Rojas *et al.* (2019, 2022) used ARIMA models for the prediction of the egg price paid to the producer, taking sorghum and maize production as auxiliary variables.

For the case of climatic events, a time series study was conducted in Peru with the monthly rainfall of two different regions using the ARIMA model; depending on the region, the ARIMA structure of the rainfall of the fluviometric stations can be different or similar (Reyes, 2014). In another study comparing energy demand in India, the Metabolic Grey model (MGM), ARIMA, their combination (MGM-ARIMA), and the back propagation neural network (BP) model were used, with the intention of applying them to policies to improve energy security. It was concluded that all four models have an accuracy of more than 95 % confidence. The most reliable model was the BP, and the MGM-ARIMA model was more accurate than the separate MGM and ARIMA models (Jiang *et al*., 2018). With these methodologies, time series information can be analyzed and explained, patterns can be identified, and variables of interest in natural, social, financial, or economic phenomena can be predicted.

MATERIALS AND METHODS

A time series of weighted wholesale white egg prices was used (Yt). As an exogenous variable, the time series of egg imports into Mexico was used (X_t). Statistical analysis was performed in R software version 4.2.0.

Weighted wholesale white egg price database

The time series contains 192 monthly national wholesale price data points per kilogram of white egg from January 2006 to December 2021. This base is constructed from the average month-end wholesale prices of white eggs in the country's main supply centers recorded in the National System of Market Information and Integration (SNIIM) for the aforementioned period (SNIIM, 2022). The weighting of the wholesale price of white eggs was based on the monthly production volume per state, i.e., the prices of supply centers in states with higher production are weighted more heavily than those in states with lower production. Production data by state are from the Agricultural, Food, and Fisheries Information Service (SIAP).

Mexican import database

The import database was constructed with information from the Economic Information System (Banco de México, 2022). This includes the sum of imports of birds' eggs with and without shells (egg yolks, fresh, dried, cooked by steaming or boiling in water, molded, frozen, or otherwise preserved). This base contains the same number of observations as the time series of weighted egg prices over the same period. The data is measured in thousands of dollars. Because of the correlation between the two variables, it was decided to include shell-on and shell-off poultry egg imports in the time-series analysis of the wholesale white egg price. The higher the number of egg imports, the lower the expected egg price.

Methodology and models

The methodology proposed by Box and Jenkins (1973) applied to ARIMA models—regression with ARIMA errors, ARIMA with interventions, and ARIMA with transfer—was used in order to find the model that best fit the white egg price series according to its Akaike index and forecast error.

ARIMA (p,d,q)(P,D,Q)s models

The ARIMA (p,d,q)(P,D,Q)s models, in their most general form, arise by merging the non-seasonal and seasonal ARIMA models:

$$\phi_p(B)\Phi_P(B^s)\nabla^d\nabla_s^D Z_t = \delta + \theta_q(B)\Theta_Q(B^s)a_t,$$

where Z_t is the response variable, δ is a constant, $\phi_p(B) = \left(1 - \phi_1 B - \phi_2 B^2 - \cdots - \phi_p B^p\right)$ is the non-seasonal autoregressive term, $\Phi_P(B^s) = \left(1 - \Phi_1 B^s - \Phi_2 B^{2s} - \cdots - \phi_p B^{Ps}\right)$ is the seasonal autoregressive term, $\theta_q(B) = \left(1 - \theta_1 B - \theta_2 B^2 - \cdots - \theta_q B^q\right)$ is the non-seasonal moving averages term, $\Theta_Q(B) = \left(1 - \Theta_1 B^s - \Theta_2 B^{2s} - \cdots - \theta_Q B^{Qs}\right)$ is the seasonal moving averages term, $\nabla^d = (1 - B)^d$ is the differencing of the non-seasonal part with d differencing, $\nabla_s^D = (1 - B^s)^D$ is the differencing of the seasonal part with D differencing, and α_t is the white noise (Tsay, 2010).

Regression with time series errors

In many applications, the relationship between two time series is particularly interesting if it leads to the consideration of a linear regression. In the standard linear regression, the errors are independent and identically distributed. However, in various applications of regression analysis, this assumption does not hold true. It is often more appropriate to assume that the errors come from a stationary second-order process with zero-mean. Since the errors can be approximated by an appropriately chosen ARMA (p,q) process, it is of particular interest to consider the following model:

$$Y_t = \alpha + \beta X_t + e_t,$$
$$e_t = \phi_0 + \sum_{i=1}^{p} \phi_i Y_{t-i} + a_t - \sum_{i=1}^{q} \theta_i a_{t-i}$$

where Y_t represents the dependent variable at the time t that is intended to be predicted, α is the coefficient of intersection in linear regression, β is the coefficient of the independent variable X_t in linear regression, and e_t reflects the discrepancy between the actual observation and the model prediction.

Within the ARMA process, ϕ_0 is the constant coefficient on the autoregressive (AR) component; ϕ_i is the coefficient of the autoregressive terms, with i varying from 1 to p; the terms Y_t are the past values of the variable over time Y; t-i is the error term over time α_t ; t is the coefficient of the moving average terms, with θ_i varying from 1 to q; and the term α_{t-i} is the past values of the error terms over time t-i (Brockwell and Davis, 2002).

ARIMA models with interventions

This methodology was developed by Box and Tiao (1975) and is used to model interventions or changes in time series. The effects of exogenous variables ξ_t (impulses or level changes) can be modeled as follows:

$$Y_t = \sum_{i=1}^{n} \frac{\omega_i(B)}{\delta_i(B)} \xi_{ti} + N_t,$$

where δ_i and ω_i represent n previously lumped parameters of the dynamic transfer of ξ_{t-i}, B is the lag operator, and N_t is the error, which is modelled as a time series model as follows:

$$N_t = \phi_0 + \sum_{i=1}^{p} \phi_i Y_{t-i} + a_t - \sum_{i=1}^{q} \theta_i a_{t-i}$$

In these models, ξ_t is considered as a deterministic or intervening variable, which is generally associated with two types of effects: impulse, taking a value of one when the impulse happens in time ξ_t and zero when the impulse does not happen:

$$\xi_t \equiv \begin{cases} 1 \; if \; t = T \\ 0 \; if \; t \neq T \end{cases}$$

and step, which takes a value of one when the level or step change occurs in time T and zero when the level change does not occur:

$$\xi_t \equiv \begin{cases} 1 \text{ if } t \geq T \\ 0 \text{ if } t < T \end{cases}$$

ARIMA models with transfer

An important type of dynamic relationship between an output Y_t that is explained by an input X_t (each measured in equispaced times) is one in which the deviations of the input and output from the appropriate mean values are related by a linear difference equation (Reinsel, 1997):

$$\begin{aligned}(1-\delta_1 B-\cdots-\delta_r B^r)Y_t &= (\omega_0-\omega_1 B-\cdots-\omega_s B^s)X_{t-b}\\ (1-\delta_1 B-\cdots-\delta_r B^r)Y_t &= \left(\omega_0 B^b-\omega_1 B^{b+1}-\cdots-\omega_s B^{b+s}\right)X_t\\ \delta(B)Y_t &= \omega(B)B^b X_t\\ \delta(B)Y_t &= \omega(B)X_{t-b}\end{aligned}$$

Alternatively, the output Y_t and input X_t can be said to be linked by a linear filter:

$$\begin{aligned}Y_t &= \upsilon_o X_t + \upsilon_1 X_{t-1} + \upsilon_2 X_{t-2} + \cdots\\ &= \upsilon(B)X_t,\end{aligned}$$

where the transfer function $\upsilon(B) = \upsilon_0 + \upsilon_1 B + \upsilon_2 B^2 + \ldots$ can be expressed as a ratio of two polynomial operators:

$$\upsilon(B) = \delta^{-1}(B)\ \omega(B)\ X_{t-b}$$

However, the problem of estimating with the model, linking an output Y_t and an input X_t, is complicated in practice by the presence of noise N_t, which corrupts the true relationship between input and output according to the following equation:

$$Y_t = \upsilon(B)\ X_t + N_t$$

where X_t and N_t are independent processes and N_t can be described as a stationary stochastic process as an ARMA (p,q) model:

$$N_t = \phi_0 + \sum_{i=1}^{p} \phi_i Y_{t-i} + a_t - \sum_{i=1}^{q} \theta_i a_{t-i}$$

In practice, it is necessary to estimate the transfer function of the linear filter describing the noise, in addition to the transfer function $\upsilon(B) = \delta^{-1}(B)\ \omega(B)\ X_{t-b}$ describing the dynamic relationship between the input and output (Box *et al*., 2016).

Akaike index

The Akaike information criterion (AIC) is defined as:

$$AIC = \frac{-2}{T} \text{In (probability)} + \frac{2}{T}\text{(number of parameters)}$$

where the likelihood function is evaluated in maximum likelihood estimates and T is the sample size (Akaike, 1974).

Cross-validation

Cross-validation generally uses three statistics to measure the performance of point forecasts: the root means square error (RMSE), the mean absolute deviation (MASE), and the mean absolute percentage error (MAPE). For step-by-step forecasts, these measures are defined as follows:

$$RMSE(\ell) = \sqrt{\frac{1}{m}\sum_{j=0}^{m-1}\left[x_{T+\ell+j} - x_{T+j}(\ell)\right]^2},$$

$$MASE(\ell) = \frac{\frac{1}{m}\sum_{j=0}^{m-1}\left|x_{T+\ell+j} - x_{T+j}(\ell)\right|}{Q},$$

$$MAPE(\ell) = \frac{1}{m}\sum_{j=0}^{m-1}\left|\frac{x_{T+j}(\ell)}{x_{T+j+\ell}} - 1\right|,$$

where m is the number of available step-by-step forecasts in the forecast sub-sample and Q is a scaling constant. Regularly, the model with the lowest value on these measures is considered the best forward forecasting model (Diebold and Mariano, 2002; Hyndman and Koehler, 2006; Kim and Kim, 2016).

RESULTS AND DISCUSSION

Series characteristics

The time series of monthly wholesale white egg prices in Mexico covers a period from January 2006 to December 2021 (192 observations). The latter presents a pattern of growth and variability with three factors: trend, seasonality, and a random component, also known as white noise. The seasonality of the wholesale white egg price series throughout the year shows that the months of February, March, and December have the highest average values for this product. The lowest price was reached in June.

Transformations and augmented Dickey-Fuller test

To achieve variance stationarity, a logarithmic transformation was applied to the egg price series. The augmented Dickey-Fuller test (Cheung and Lai, 1995) examines the hypotheses H_0 (series has a unit root) and H_a (series does not have a unit root) at a significance level of $\alpha = 0.05$ to determine whether a series is stationary or not. By using the adf.test() function in the R statistical package on the transformed series, a value of $p = 0.1706$ was obtained, which is greater than α. Therefore, H_0 is not rejected, and the series is concluded to have a unit root. As a result, the series needed to be differentiated and then retested.

Taking the first difference, a stationary series in variance and level is obtained, which is confirmed by the augmented Dickey-Fuller test. When applying the test in R, $p = 8.2879 \times 10^{-12}$, which is less than the significance level of $\alpha = 0.05$. Therefore, H_0 is rejected, and the series is found to be stationary.

Autocorrelation (ACF) and partial autocorrelation (PACF) graphs

ACF and PACF charts are useful tools for analyzing time series properties and determining the appropriate ordering of an ARIMA model (Box and Pierce, 1970; Velicer, 1976). The graphs of the series with the logarithmic transformation of the wholesale white egg price in Mexico, obtained using the ggAcf() function of the forecast package in R (Figure 1), show a significant correlation at lag 12. This suggests that the series has a seasonal component, as the correlation is high at 12-month intervals.

Selection of the candidate ARIMA models

Based on the ACF and PACF plots of the time series, ARIMA models (Table 1) are proposed, which include regression models with ARIMA errors, ARIMA with interventions, and ARIMA with transfers.

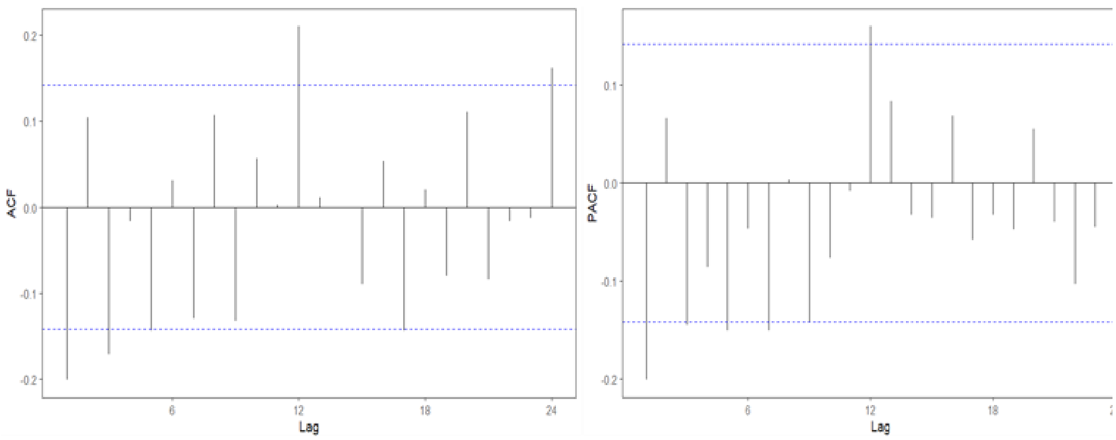

Figure 1. Autocorrelation (ACF) and partial autocorrelation (PACF) graphs for the determination of the model.

Table 1. Proposed ARIMA models to analyze the time series.

Models
ARIMA (4,1,3)(1,0,1)[12]
ARIMA (3,1,3)(1,0,1)[12]
ARIMA (3,1,2)(1,0,1)[12]
ARIMA (3,1,1)(1,0,1)[12]
ARIMA (3,1,0)(1,0,1)[12]
ARIMA (2,1,3)(1,0,1)[12]
ARIMA (2,1,2)(1,0,1)[12]
ARIMA (2,1,1)(1,0,1)[12]
ARIMA (2,1,0)(1,0,1)[12]
ARIMA (1,1,3)(1,0,1)[12]
ARIMA (1,1,2)(1,0,1)[12]
ARIMA (1,1,1)(1,0,1)[12]
ARIMA (1,1,0)(1,0,1)[12]
ARIMA (0,1,3)(1,0,1)[12]
ARIMA (0,1,2)(1,0,1)[12]
ARIMA (0,1,1)(1,0,1)[12]

ARIMA model adjustment

Estimating the coefficients of the candidate ARIMA models

To estimate the parameters of the proposed ARIMA models, the arima() function from the forecast package in R was used. Models with parameters that did not meet the 0.05 significance level were discarded. There were only four models with all significant coefficients: ARIMA (3,1,1)(1,0,1)[12], ARIMA (1,1,3)(1,0,1)[12], ARIMA (1,1,0)(1,0,1)[12] and ARIMA (0,1,1)(1,0,1)[12].

Diagnosis of candidate ARIMA models

To check for white noise in the residuals, the ACF plot and the Ljung-Box test, which measure the correlation between the values of the series at different lags where the hypotheses are tested, were used for a value $\alpha = 0.05$, H_0 (the residuals are white noise), and H_a (the residuals are not white noise) (Ljung and Box, 1978). To verify normality in the residuals, the Jarque-Bera test was used, which compares the skewness and kurtosis of the series to those of a theoretical normal distribution with the hypotheses H_0 (there is normality in the residuals) and H_a (there is no normality in the residuals), for a value $\alpha = 0.05$ (Jarque and Bera, 1981).
The p values showed that there is insufficient evidence to reject the null hypothesis in the case of the Ljung-Box test, as the p values for all four models are greater than 0.05. Conversely, the Jarque-Bera test rejects the null hypothesis of normality in all models. None of the models that did not meet the assumption of normality in the residuals was discarded, and therefore, they were still considered for robustness.

Best ARIMA model by the Akaike index

The selection criterion is to choose the one with the lowest Akaike index score. In this case, the ARIMA (1,1,3)(1,0,1)[12] model was the best, with a value of -329.3, followed by ARIMA (3,1,1)(1,0,1)[12] with -328.43, ARIMA (1,1,0)(1,0,1)[12] with -326.36, and ARIMA (0,1,1)(1,0,1)[12] with -324.74.
The ARIMA (1,1,3)(1,0,1) model[12] can be written as follows:

$$(1-B)Y_t = \frac{(1+0.7911B-0.2539B^2+0.2266B^3)(1+0.9310B)}{(1-0.5257B)(1-0.9917B)}a_t,$$

where $\{a_t\}$ is white noise with zero mean and $\sigma^2 = 0.0094$, $\theta_1 = 0.7911$, $\theta_2 = 0.2539$, $\theta_3 = 0.2266$, $\theta_1 = -0.9310$, $\phi_1 = 0.5257$, and $\Phi_1 = 0.5257$, and $\Phi_1 = 0.9917$.

Best ARIMA model by the forecast error

For the selection of the best ARIMA model according to the estimation of the errors (RMSE, MAPE, and MASE) through cross-validation, the period from January 2006 to December 2018 was considered the training set. The coefficients of the models already selected up to this point were re-estimated, and the following three years were forecast. The forecasts were then compared with the original series to measure their accuracy. According to the methodology, the best model is the ARIMA (1,1,0)(1,0,1)[12], which has lower values in all adjustment metrics.

$$(1-B)\,ln(Y_t)=\frac{(1+0.9126B)}{(1+0.2505B)(1-0.9870B)}a_t,$$

where $\{a_t\}$ is white noise with zero mean and $\sigma^2 = 0.0098$, θ_1 = -0.9126, ϕ_1 = -0.2505, and Φ_1 = 0.9870.

Forecasting in ARIMA models

The best models, according to the Akaike index and forecast error adjustment metrics, were ARIMA (1,1,3)(1,0,1)[12] and ARIMA (1,1,0)(1,0,1)[12], respectively. The forecast for both models were obtained using the R software (Figure 2).

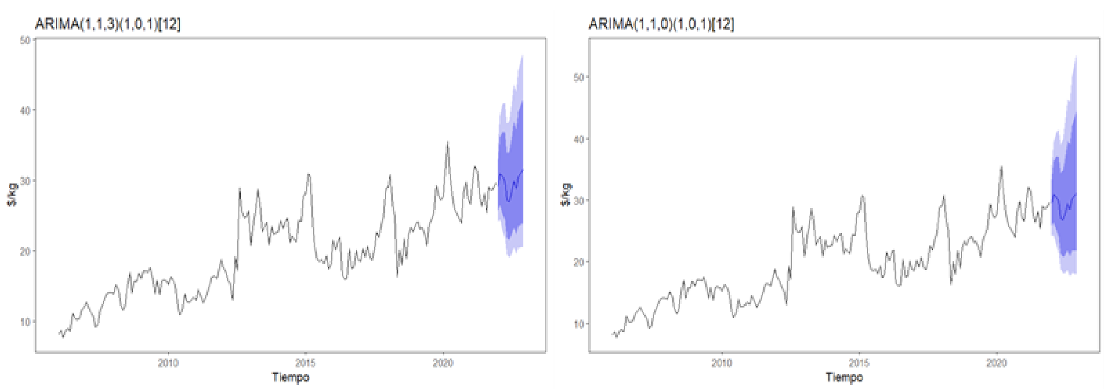

Figure 2. ARIMA model forecasting.

Regression model adjustment with ARIMA errors

Selection of candidate regression models with ARIMA errors

The same ARIMA models (Table 1) were proposed for regression models with ARIMA errors as found in the ACF and PACF plots (Figure 1). These models were used to estimate the error of a regression that included an exogenous variable, in this case the square root of egg imports into Mexico. These models were used to estimate the error of a regression that included an exogenous variable, in this case the square root of egg imports into Mexico.

Estimating coefficients of regression models with candidate ARIMA errors
In this case, the same logic was followed as in the ARIMA models, eliminating the models with parameters not significant at the level of 0.05 Only two models had all significant coefficients (regression with ARIMA (1,1,0)(1,0,1) errors[12] and regression with ARIMA (0,1,1)(1,0,1) errors[12]).

Diagnosis of regression models with candidate ARIMA errors
The same procedure as in the ARIMA models was used to diagnose the residuals and ensure that the model assumptions were met. Both models met the white noise criterion but failed to meet the residual normality criterion. As with the ARIMA models, the pair of models were kept to assess their accuracy.

Best regression model with ARIMA errors by Akaike Index
The best regression model with ARIMA errors, according to the Akaike criterion, is the regression model with ARIMA error (1,1,0)(1,0,1)[12], which presented the lowest value (-335.5), surpassing the ARIMA (1,1,0)(1,0,1)[12], which presented a value of -333.84. The regression model with ARIMA (1,1,0)(1,0,1) errors[12] can be written as follows:

$$(1-B)\ln(Y_t) = 0.0023\sqrt{X_t} + N_t$$

$$N_t = \frac{(1+0.8961B)}{(1+0.3215B)(1-0.9840B)}a_t,$$

where $\{a_t\}$ is white noise with zero mean and σ_2 = 0.0093, θ_1 = -0.8961, ϕ_1 = -0.3215, Φ_1 = 0.9840, and X_t = eggs imports in Mexico.

Best regression model with ARIMA errors by the forecast error
The best model according to the forecast error criterion is the regression model with ARIMA error (1,1,0)(1,0,1)[12], which presented the lowest value in all adjustment metrics, coinciding with the best model found by the Akaike index.

Forecasting in regression models with ARIMA errors
In this step, an ARIMA model with interventions to the exogenous variable was used to forecast the dependent variable based on the regression model. The ARIMA (0,1,3) error model was associated with the dynamic intervention model that best fitted the egg import time series data. The ARIMA (1,1,0)(1,0,1)[12] is the best error model associated with the regression based on the Akaike index and forecast error metrics (Figure 3)

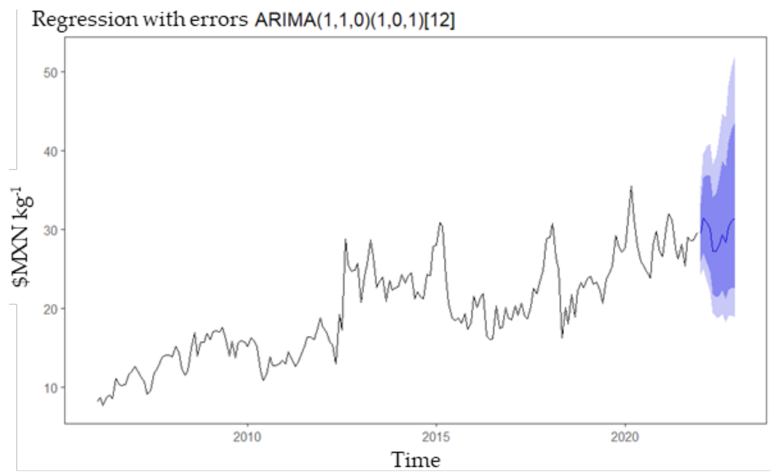

Figure 3. Forecasting the regression model with ARIMA errors.

ARIMA model adjustment with interventions

Identification of interventions

To identify changes in the series' level, the tso() function from the tsoutliers package in R was applied to the log-transformed series. Three interventions that changed the level of the series were detected in July and August 2012, as well as in May 2018. These interventions were modeled using dummy variables distributed in vectors that had values of zero before and one after the intervention.

The seasonality effect of the months of the year with the greatest variation in egg prices was also considered in the analysis. February, March, April, and June were discovered to have the greatest effect, and they were modelled with dummy variables that indicated one when the month occurred and zero when it did not.

Selection of ARIMA models with candidate interventions

The same ARIMA models identified (Table 1) were proposed according to the ACF and PACF plots (Figure 1), incorporating the effects of the interventions. In some models, the June effect was omitted because it was not significant.

Coefficient estimation for ARIMA models with candidate interventions

The same logic was followed as in the previous models. Only four models had all significant coefficients (ARIMA (3,1,2)(1,0,1)[12], ARIMA (2,1,3)(1,0,1)[12], ARIMA (1,1,0)(1,0,1)[12] and ARIMA (0,1,1)(1,0,1)[12] with interventions).

ARIMA model diagnosis with candidate interventions

The same procedure as in previous models was used to diagnose the residuals and ensure that they met the model's assumptions. In this case, all models met the criteria for residual normality and white noise.

Best ARIMA model with interventions by the Akaike index

According to the Akaike criterion, the best model was ARIMA (0,1,1)(1,0,1)[12] with interventions, which showed a value of -371.93, followed by ARIMA (1,1,0)(1,0,1)[12] with -371.81, ARIMA (3,1,2)(1,0,1)[12] with -371.23, and ARIMA (1,1,0)(1,0,1)[12] with -370.93. The ARIMA (0,1,1)(1,0,1)[12] model with interventions can be writte as follows:

$$(1 - B)\ln(Y_t) = 0.3793\ \xi_{1t} + 0.4324\ \xi_{2t} - 0.3111\ \xi_{3t} + 0.0826\ \xi_{4t} + 0.0790\ \xi_{5t} + 0.0712\ \xi_{6t} + N_t$$

$$N_t = \frac{(1 + 0.1701B)(1 + 0.9353B)}{(1 - 0.9866B)} a_t,$$

$$\xi_{1t} = \begin{cases} 0 \ \mathit{if}\ T < 78 \\ 1 \ \mathit{if}\ T \geq 78 \end{cases}$$

$$\xi_{2t} = \begin{cases} 0 \ \mathit{if}\ T < 80 \\ 1 \ \mathit{if}\ T \geq 80 \end{cases}$$

$$\xi_{3t} = \begin{cases} 0 \ \mathit{if}\ T < 149 \\ 1 \ \mathit{if}\ T \geq 149 \end{cases}$$

$$\xi_{4t} = \begin{cases} 0 \ \mathit{if}\ T \neq feb \\ 1 \ \mathit{if}\ T = feb \end{cases}$$

$$\xi_{5t} = \begin{cases} 0 \ \mathit{if}\ T \neq mar \\ 1 \ \mathit{if}\ T = mar \end{cases}$$

$$\xi_{6t} = \begin{cases} 0 \ \mathit{if}\ T \neq abr \\ 1 \ \mathit{if}\ T = abr \end{cases}$$

where, according to the Jarque-Bera test carried out on the residuals, $\{a_t\}$~N (0,0.0076), θ_1 = -0.1701, θ_1 = -0.9353, and Φ_1 = 0.9866.

Best ARIMA model with interventions by the forecast error

According to the forecast error criterion, the best model was the ARIMA (3,1,2)(1,0,1)[12] with interventions, which presented the lowest value in all metrics. The model can be written as follows:

$$\begin{aligned}(1 - B)\ln(Y_t) = {} & 0.4194\xi_{1t} + 0.4241\xi_{2t} - 0.2950\xi_{3t} \\ & + 0.0815\xi_{4t} + 0.0814\xi_{5t} + 0.0711\xi_{6t} \\ & - 0.0564\xi_{7t} + N_t\end{aligned}$$

$$N_t = \frac{(1 + 0.5628B - 0.9999B^2)(1 + 0.8717B)}{(1 - 0.3762B + 0.8527B^2 + 0.2158B^3)(1 - 0.9437B)} a_t,$$

$$\xi_{1t} = \begin{cases} 0 \ \mathit{if}\ T < 78 \\ 1 \ \mathit{if}\ T \geq 78 \end{cases}$$

$$\xi_{2t} = \begin{cases} 0 & if\ T < 80 \\ 1 & if\ T \geq 80 \end{cases}$$

$$\xi_{3t} = \begin{cases} 0 & if\ T < 149 \\ 1 & if\ T \geq 149 \end{cases}$$

$$\xi_{4t} = \begin{cases} 0 & if\ T \neq feb \\ 1 & if\ T = feb \end{cases}$$

$$\xi_{5t} = \begin{cases} 0 & if\ T \neq mar \\ 1 & if\ T = mar \end{cases}$$

$$\xi_{6t} = \begin{cases} 0 & if\ T \neq abr \\ 1 & if\ T = abr \end{cases}$$

$$\xi_{7t} = \begin{cases} 0 & if\ T \neq jun \\ 1 & if\ T = jun \end{cases}$$

where, according to the Jarque-Bera test, $\{a_t\}$~N (0,0.0075), θ_1 = -0.5628, θ_2 = -0.9999, θ_1 = -0.8717, ϕ_1 = 0.3762, ϕ_2 = -0.8527, ϕ_3 = -0.2158, and Φ_1 = 0.9437.

Forecasting ARIMA models with interventions

According to the Akaike index and forecast error metrics, ARIMA (0,1,1)(1,0,1)[12] and ARIMA (3,1,2)(1,0,1)[12] were found to be the best models associated with the error of the ARIMA (0,1,1)(1,0,1)[12] interventions. Model forecasts were obtained in R software (Figure 4).

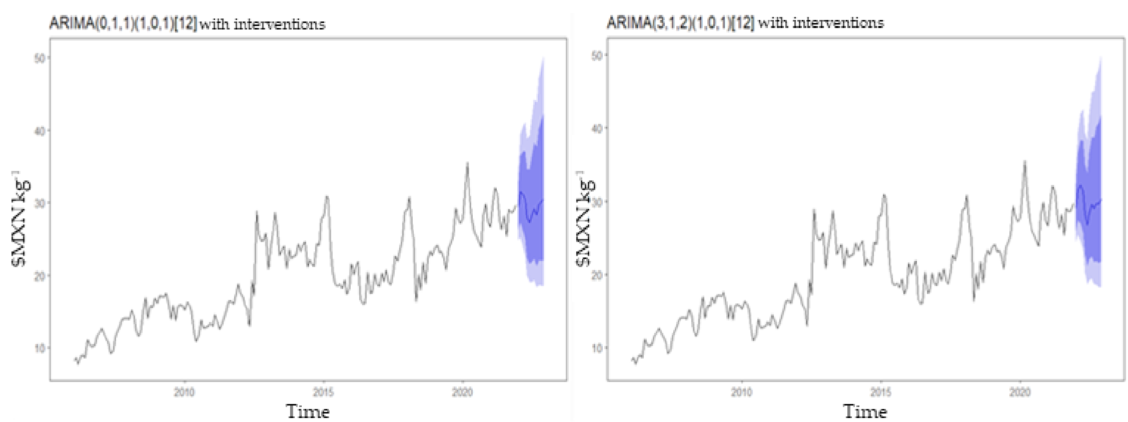

Figure 4. ARIMA model forecasting with interventions.

ARIMA model adjustment with transfer

Correlation graph of the dependent variable and predictor variable

The cross-correlation between the dependent variable (wholesale white egg price) and the predictor (egg imports) indicated that both variables are contemporaneous since there is a significant peak at lag zero. This indicates that there is no effect of lags of the predictor variable on the dependent variable, implying that the relationship between the variables does not change over time. Therefore, the analysis only considers the effect of the predictor variable without lags, which is precisely the definition of regression models with ARIMA errors. The adjustment results of the ARIMA models with transfer are the same as those of the linear regression model with ARIMA errors.

Comparison of methodologies according to Akaike index and forecast error metrics. According to the Akaike index (Table 2), the best model was the ARIMA (0,1,1)(1,0,1)[12] with interventions, followed by the regression models with ARIMA and ARIMA with transfer errors, which had the same adjustment, because they turned out to be the same model since the wholesale white egg price series and imports are contemporaneous.

Table 2. Comparison of methodologies by Akaike index.

	ARIMA	Regression with ARIMA errors	ARIMA with interventions
Akaike index	-329.3	-335.5	-371.9
Ljung-Box test *p*-value	0.9974	0.7763	0.7781
Jarque-Bera test *p*-value	6.929×10^{-6}	2.051×10^{-7}	0.8992
Variance	0.0094	0.0093	0.0076

According to the forecast errors (Table 3), the most accurate models were the regression model with ARIMA (1,1,0)(1,0,1) error[12] and the ARIMA (1,1,0)(1,0,1) error[12] with transfer since they are equivalent models. In both comparisons, only the ARIMA models with interventions meet the assumptions of white noise and normality in the residuals, in addition to having lower variance.

Luis-Rojas *et al*. (2019) found as the best model for egg price prediction the ARIMA (0,1,1)(1,0,1)[12], with an Akaike index of -332.538 and a variance of 0.0104. According

Table 3. Comparison of methodologies according to forecast error.

	ARIMA	Regression with ARIMA errors	ARIMA with interventions
RMSE	3.946	3.698	4.573
MAPE	10.91	10.12	13.04
MASE	0.8936	0.8245	1.071
Ljung-Box test *p*-value	0.7543	0.7763	0.8624
Jarque-Bera test *p*-value	2.719×10^{-11}	2.051×10^{-7}	0.8358
Variance	0.0098	0.0093	0.0075

to these authors, the model's short-term predictions deviate by 12.38 % from the observed data. However, they do not report specific data on their forecast errors. This result largely coincides with that found in the present study, since among the best prediction models is the ARIMA (0,1,1)(1,0,1)[12] with a slightly higher Akaike index (-329.3), which can be explained by the size of the series.

Furthermore, Luis-Rojas *et al.* (2022) obtained a time series model to forecast nominal monthly white egg prices paid to the producer in Mexico using transfer function models (TFM), evaluating their relationship with average rural sorghum prices. This model yielded an Akaike index of -352.97 and a variance of 0.0098. As can be seen, the ARIMA (0,1,1)(1,0,1)[12] model with interventions has a lower Akaike index (-371.9) and variance (0.0075).

CONCLUSIONS

The regression model with ARIMA errors turned out to be the same as the ARIMA with transfer, since the latter is a modification of the former and the lags of the predictor variable (egg imports into Mexico) had no significant effect on the response variable (wholesale white egg price in Mexico). According to Akaike's index, the model that presented the best adjustment was the ARIMA (0,1,1)(1,0,1)[12] with interventions, followed by the regression models with ARIMA errors and ARIMA with transfer. In the evaluation of forecast error, the best models were the regression models with ARIMA (1,1,0)(1,0,1)[12] and ARIMA (1,1,0)(1,0,1)[12] errors with transfer, followed by ARIMA with interventions. These models made it possible to generate reliable predictions of the price of white eggs in Mexico, which can help generate strategies that benefit in the short, medium, and long term.

According to the Akaike index and forecast error metrics, the ARIMA models with interventions presented low variance and met the assumptions of white noise and normality in the residuals. Furthermore, it can be concluded that the use of the ARIMA model with interventions, ARIMA with transfers, and regression with ARIMA errors improves the forecasting of the ARIMA model for the wholesale price of white eggs.

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ANALYSIS FOR MULTIPLE RESPONSES IN A COMPLETELY RANDOMIZED EXPERIMENTAL DESIGN

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ABSTRACT

Multiple responses are often generated in agricultural and forestry research. For example, the moisture content, fatty acids, carbohydrates, size, diameter, length, shape, and hardness, among other characteristics, are measured in cottonseeds. Multivariate analysis of variance (MANOVA) can be useful for multiple response analysis when differences in treatment effects are to be determined. However, the performance of current *post hoc* tests in this context is not satisfactory due to the limitations of the available methods or because they are difficult to use for non-statistician researchers. Furthermore, this methodology requires the assumptions of multivariate normality and homogeneity of variance and covariance matrices, assumptions that are difficult to verify if the sample size is small. This research proposes an alternative analysis to test the hypothesis of equality of effects between treatments and *post hoc* tests in the case of multiple responses. An asymptotic result is demonstrated for the random variable generated in the proposal for the case of uncorrelated normal variables, and the case for correlated normal random variables is left open. A simulation study shows that the performance of the proposal with small samples is satisfactory in terms of power and that it has advantages compared to MANOVA. Furthermore, the methodological approach allows for *post hoc* testing in the case of multiple responses in the completely randomized experimental design.

Keywords: ANOVA, MANOVA, assumptions, data transformation, Euclidean norm.

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INTRODUCTION

The generation of multiple responses is common in research in a variety of areas. For example, Pérez-López *et al*. (2014) presented a study of fava bean cultivars where the following responses were recorded: plant height, number of branches, number of flower nodes, number of pods per plant, pod weight per plant, number of seeds per pod and per plant, total seed weight per plant, number of clean seeds per plant, and weight of clean seed per plant of 100 seeds and of spotted seed per plant. In other research, measurements of weight, color, texture, protein, fat, and vitamin content were obtained from a portion of chicken meat (Sosnówka-Czajka *et al*., 2023); the number of bacteria, pH, and fiber and vitamin content were obtained from cactus

(El-Mostafa *et al*., 2014; Hernández-Anguiano *et al*., 2016); and moisture content, fatty acids, carbohydrates, size, diameter, length, shape, and hardness were measured from cottonseed (Anitha *et al*., 2022).

Multivariate analysis of variance (MANOVA) is one methodology used to evaluate the hypothesis of equality of effects between two or more treatments when there are many responses. However, when MANOVA rejects the hypothesis of equal treatment effects, there are no satisfactory alternatives for *post hoc* testing. The methodology developed by Seo *et al.* (1994) was tested for a limited number of treatments and variables. This methodology requires the assumptions of multivariate normality, homogeneity of covariance matrices. Unfortunately, these assumptions are difficult to verify if the sample size is small (Hair, 1999; Dattalo, 2013). Moreover, such a methodology is difficult to implement for non-statistician researchers. Warne *et al*. (2012) found that 5 out of 62 articles that used MANOVA in educational psychology journals had correctly applied *post hoc* procedures. Furthermore, Warne (2014) screened the top three psychology journals and found that, in 58 articles, researchers used MANOVA between 2009 and 2013; however, none of these articles used *post hoc* procedures.

Much of the statistical methodology proposed for comparing multiple mean vectors is based on the T^2_{max} statistic (Seo, 2002; Nishiyama *et al*., 2014). However, as argued by Nishiyama and Seo (2013) and Nishiyama *et al*. (2014), finding the distribution of the test statistic is difficult, even in the simplest cases of pairwise comparison of vector means, assuming normality. Hence, the upper quantiles of the statistic T^2_{max} have been determined only for particular cases. For example, assuming normality in the data, Nishiyama and Seo (2013) determined the 0.9, 0.95, and 0.99 quantiles of the distribution of the T^2_{max} statistic as part of their proposed methodology for testing four vectors of correlated means.

In this context, the present research paper proposes an alternative analysis for the determination of between-treatment effects and *post hoc* tests for the case of multiple responses.

MATERIALS AND METHODS

Multiple response data generated in a completely randomized experimental design (CRD) can be modeled by the following: $\boldsymbol{Y}_{ij} = \boldsymbol{\mu} + \boldsymbol{\tau}_i + \boldsymbol{e}_{ij}$, where each component of the model is a p-dimensional vector: $\boldsymbol{Y}_{ij} = (Y_{ij1}, ..., Y_{ijp})^t$ is the random vector of response variables for the j-th repetition of the i-th treatment $i =1,..., t$, $j = 1, ... r$, whose element Y_{ijk} corresponds to the k-th random variable, $k = 1, ..., p$; $\boldsymbol{e}_{ij} = (e_{ij1}, ..., e_{ijp})^t$ is the vector of random errors; $\boldsymbol{\mu} = (\mu_1, ..., \mu_p)^t$ is the vector of overall means; and $\boldsymbol{\tau}_i = (\tau_{i1}, ..., \tau_{ip})^t$ is the vector of effects of the i-th treatment. In practice, $\boldsymbol{\mu}$ and $\boldsymbol{\tau}_i$ are unknown parameters (Rencher and Christensen, 2012).

The set of hypotheses used in the MANOVA is as follows:

H_0: $\tau_1 = \tau_2 = .. = \tau_t$ vs. = H_a: $\tau_i \neq \tau_j$, for at least a $i \neq j$.

Johnson and Wichern (2007) stated that, in order for the data to meet the basic assumptions for the MANOVA result to be reliable, the observations must be random samples of size r of treatment i, the random samples of the treatments must be independent, and each treatment must have a multivariate normal distribution with a common variance and covariance matrix for all treatments, i.e: $\boldsymbol{Y}_{ij} \sim N_p\ (\boldsymbol{\mu}_i, \boldsymbol{\Sigma})$, where $\mu_i = (\mu_1 + \tau_{i1}, ..., \mu_p + \tau_{ip})^t$.

If H_0 is rejected, it is necessary to identify which treatments have different effects from each other; for this purpose, vector mean comparison methods are used. A very popular alternative is the Hotelling T^2 method by applying the Bonferroni correction. However, this method is very conservative (Dattalo, 2013). Another is the generalized Tukey conjecture, developed by Seo *et al*. (1994) and Seo and Nishiyama (2008), which is a generalization of the univariate Tukey-Kramer methodology. In the procedure, confidence intervals are generated for the differences by pairs of mean vectors. This proposal has the limitation that it is only used for a maximum of four treatments, vectors with five variables, and 60 degrees of freedom (with $\upsilon = N - p - 1$, degrees of freedom).

ANOVA is another methodology to determine treatment effects with a simpler model. For example, the CRD model, $Y_{ij} = \mu + \tau_i + e_{ij}$, which is similar to MANOVA; only the components are scalar, and its basic assumptions are independence, normality, and homoscedasticity (Montgomery, 2004). When any of the assumptions are not met, methods can be used to transform the data, such as the Box and Cox (1964) methodology, although Driscoll (1996) and Salkind (2010) agree that ANOVA is robust to non-normality of the data.

When the hypothesis of equality of treatments is rejected in an ANOVA, it is necessary to identify which treatments cause the difference. For this purpose, comparisons of means are carried out. Montgomery (2004) and Hinkelmann and Kempthorne (2005) mention that the main *post hoc* methods for such comparisons are the Fisher's least significant difference (LSD), Tukey's honest significant difference (HSD), Dunnett's least significant difference (LSD), Duncan's multiple range, and the Student-Newman-Keuls (SNK) test. It should be noted that ANOVA has fewer limitations than MANOVA, as well as the development of several tests for comparison of means; however, ANOVA is not designed to analyze data with multiple responses. As a result, an alternate methodology is proposed for data from three or more treatments with various responses gathered through experimental designs.

Methodological proposal

The proposal is to reduce each vector of response variables to a scalar in order to obtain data that can be analyzed by means of an ANOVA and, subsequently, by means of a *post hoc* method to make a comparison of means.

It is proposed that each variable of the p-vector Y_{ij} be transformed with the quadratic function of the Euclidean norm as follows:

$$X_{ij} = \boldsymbol{Y}_{ij}^t \boldsymbol{Y}_{ij} = \sum_{k=1}^{p} Y_{ijk}^2 \qquad (1)$$

where Y_{ijk} is the k-th random variable ($k = 1, ..., p$) from vector $\mathbf{Y}_{ij}$ (Table 1).

Table 1. Use of the Euclidean norm in the data set.

Treatment	Variable 1	...	Variable p		Use of the square of the norm
	y_{111}	...	y_{11p}		$y_{t21}{}^2 + ... + y_{11p}{}^2 = \lVert y_{11} \rVert^2 = x_{11}$
1	y_{121}	...	y_{12p}		$y_{121}{}^2 + ... + y_{12p}{}^2 = \lVert y_{12} \rVert^2 = x_{12}$
	$\vdots$	$\vdots$	$\vdots$		$\vdots$
	y_{1r1}		y_{1rp}	$\rightarrow$	$y_{1r1}{}^2 + ... + y_{1rp}{}^2 = \lVert y_{1r} \rVert^2 = x_{1r}$
$\vdots$	$\vdots$	$\vdots$	$\vdots$		$\vdots$
	y_{t11}	...	y_{t1p}		$y_{t11}{}^2 + ... + y_{t1p}{}^2 = \lVert y_{11} \rVert^2 = x_{t1}$
t	y_{t21}	...	y_{t2p}		$y_{t21}{}^2 + ... + y_{t2p}{}^2 = \lVert y_{12} \rVert^2 = x_{t2}$
	$\vdots$	$\vdots$	$\vdots$		$\vdots$
	y_{tr1}		y_{trp}		$y_{tr1}{}^2 + ... + y_{trp}{}^2 = \lVert y_{1r} \rVert^2 = x_{tr}$

By transforming the data with multiple respBy transforming the data with multiple responses to a scalar value, a sequence of independent random variables X_{ij}, is generated, which can be analyzed by means of an ANOVA. Each of the p characteristics is obtained from the same object, so they may correlate with each other. However, the sequence of variables X_{ij} (Equation 1) and referring to the treatment i in its repetition j, can be considered independent because, *a priori*, the researcher must ensure the independence of them by randomization.

Now, note that X_{ij} it is a sum of random variables, so the following central limit theorem for the sum of random variables can be applied:

Theorem 1: Let $W_1, W_2, ..., W_n$ be a sample of n independent random variables with distribution functions $F_1, F_2, ..., F_n$, respectively, such that $E(W_i) = \mu_i$ and $Var(W_i) = \sigma_i^2$ for $i = 1, ..., n$, and $s_n^2 = \sum_{i=1}^{n} \sigma_i^2$, then:

$$S_n^* = s_n^{-1} \sum_{i=1}^{n} (W_i - \mu_i) \xrightarrow{d} Z,$$

where $Z \sim N(0, 1)$ provided that the F is absolutely continuous with density function f_i, such that the following, known as the Lindeberg condition, is satisfied:

$$\lim_{n\to\infty} s_n^{-2}\sum_{i=1}^{n}\int_{|w-\mu_i|>\epsilon s_n}(w-\mu_i)^2 f_i(w)dw = 0,$$

As the Lindeberg condition is met, the vector size (p) is sufficiently large, the variances of X_{ij} are homogeneous for all i, and the conclusions obtained from the ANOVA will be valid. The expression "large enough" is controversial and should be taken with caution, because whether certain sample sizes are considered "large enough" depends on the shape of the original distribution (Correa-Londoño and Castillo-Morales, 2000). Although there are potentially many multivariate distributions, such that the vector p-variate Y_{ij} under the transformation (Equation 1) can meet the above conditions, the most typical case will be explored.

Case 1. Uncorrelated normal variables (theoretical result)

Since MANOVA works under the assumptions of multivariate normality and homogeneity of variances and covariances, these assumptions will be used as a starting point to apply the methodological proposal.

Assuming that the vector $\boldsymbol{Y}_{ij}$ has $N_p(\boldsymbol{\mu}_i, \sigma_i^2\, \boldsymbol{I}_p)$ distribution, the random variable generated from the squared function of the norm $X_{ij} = Y_{ij}^t\, Y_{ij} = \sum_{k=1}^{p} Y_{ijk}^2$ has a non-central chi-squared distribution, with mean $p + \lambda$ and variance $2(p + 2\lambda)$, for $p > 0$ which specifies the degrees of freedom and $\lambda \geq 0$ which is the non-centrality parameter (Casella, 2008):

$$\boldsymbol{Y}_{ij}^t \boldsymbol{Y}_{ij}\sigma^{-2} \sim \chi_p^2(\lambda), \lambda = 0.5\boldsymbol{\mu}_i^t \boldsymbol{\mu}_i\, \sigma^{-2}.$$

Even if the same $\boldsymbol{Y}_{ij}$ variance is assumed for all i, X_{ij} have different variances because they depend on the mean of each treatment. Under this scenario, the random variables X_{ij} do not follow a normal distribution and do not have homogeneous variances. Therefore, if X_{ij} are used, the ANOVA results will not be valid. However, if p is sufficiently large, X_{ij} may converge to the normal distribution, so it would be feasible to use the proposed methodology in this case.

Convergence demonstration for the case of uncorrelated variables

Let $X_{ij} = \sum_{k=1}^{p} Y_{ijk}^2$ be a random a random variable with $E(Y_{ijk}^2) = \mu_{ik}$ and $Var(Y_{ijk}^2) = a_{ik}^2$ it can be assumed that there exists a constant a, such that: $|a_{ik}| \leq a$, since a_{ik} depends on μ_{ik} and σ^{-2}, so this assumption is reasonable for Case 1. On the other hand, $\sum_{k=1}^{p} a_{ik}^2 \to \infty, p \to \infty$, since the variances are always positive.

Under these considerations it can be seen that

$$s_p^{-2}\sum_{k=1}^{p}\int_{\left|Y_{ijk}^2\right|>\varepsilon s_p}\left(Y_{ijk}^2-\mu_{ik}\right)^2 f_Y\left(y_{ijk}^2\right)dy \le a^2 s_p^{-2}\sum_{k=1}^{p}P\left(\left|Y_{ijk}^2-\mu_{ik}\right|>\varepsilon s_p\right),$$

where $\mu_{ik} = E\left(Y_{ijk}^2\right)$.

Applying Chebyshev's inequality:

$$\le a^2 s_p^{-2}\sum_{i=1}^{p}Var\left(Y_{ijk}^2\right)\varepsilon^{-2}s_p^{-2}$$

$$\le a^2\varepsilon^{-2}s_p^{-2} \to 0, p\to\infty$$

Therefore, the Lindeberg condition is hold, and so

$$X_{ij}\dot{\sim}N\left(\sum_{i=1}^{t}\left(p+0.5\,\mu_i^t\mu_i\sigma^{-2}\right), 2\sum_{i=1}^{t}\left(p+\mu_i^t\mu_i\sigma^{-2}\right)\right),$$

if p es is large enough.

Furthermore, under the null hypothesis, the variances are homogeneous and, therefore, p is sufficiently large and X_{ij} meet the assumptions of ANOVA.

Case 2. Correlated variables

If the vector $\boldsymbol{Y}_{ij}$ has a distribution N_p ($\boldsymbol{\mu}_i$, $\boldsymbol{\Sigma}$), the distribution of the random variable is not known $X_{ij} = \boldsymbol{Y}_{ij}^t\,\boldsymbol{Y}_{ij} = \sum_{k=1}^{p} Y_{ijk}^2$ and remains open. However, in this case, a simulation study was conducted to examine the performance of the proposal in terms of MANOVA.

Performance assessment of the proposal for Case 1

Sometimes, the sample size does not need to be so large to obtain satisfactory convergence results, so a simulation study is presented to evaluate the proposal with sample sizes that usually appear in practice in the case of a CRD. To assess the power of the ANOVA using the transformed data, a Monte Carlo simulation study was performed, using 2000 replicates (B) and a significance level of 0.05, under the assumption that the multiple responses come from a multivariate normal distribution. The simulation study was carried out with R software version 4.3.2 (R Core Team, 2023). The parameters to be set in the simulation of multivariate normal distribution data were: 1) mean vectors for each treatment, where the main vector will bee μ_1 and from which the differences between mean vectors were generated; 2) the variance matrix, for which uncorrelated variable matrices were considered ($\Sigma = I_p$); 3) number of treatments, t = 3, 5, 7; 4) number of variables: p = 3, 5, 7; and 5) number of replicates per treatment, r = 4, 8, 12, 16.

The performance of the proposed methodology through power estimation was evaluated as follows: 1) select p, t y r; 2) generate a random sample with multivariate normal distribution for each treatment, with vector of means μ_i and a common variance matrix Σ in all treatments; 3) obtain the transformed variables X_{ij} from the sample; 4) perform an ANOVA on the X_{ij} and obtain the degrees of freedom of the treatments ($glTrat$), the degrees of freedom of the error ($glTrat$) and the mean square of the error (CM_E); 5) calculate the means of each treatment as: $p + \hat{\mu}_i^t \hat{\mu}_i$; 6) calculate the average of the treatment means ($\hat{\bar{\mu}}$) ; 7) calculate the estimator of the non-centrality parameter, $\hat{\lambda} = r \sum_{i=1}^{t} (\hat{\mu}_i - \hat{\bar{\mu}})^2 \; CM_E^{-1}$; 8) obtain pf as the cumulative distribution function of the non-central F $\left(F_{glError}^{glTrat}(\hat{\lambda})\right)$; 9) obtain $F_{critical}$ as the quantile 1 - pf of the central F distribution $F_{glError}^{glTrat}$; 10) estimate the power of the ANOVA as assessed 1 - pf in the $F_{critical}$; 11) repeat B times steps 2–10; and 12) estimate the power as the proportion of times it was rejected in the H_0 simulation.

Comparison of MANOVA results with the proposal using ANOVA

To compare the proposed methodology to the MANOVA, data with multivariate normal distribution were simulated by varying the following parameters of interest:

Vectors of means (μ´*s*)

To calculate the distance between the means of the elements in μ_1, random numbers drawn from the uniform distribution using the *runif* function in R (R Core Team, 2023) were considered, so that it μ_1 was contained in one of the following intervals: [1, 10] or [50, 100]. For example, in the case of $\mu_1 \in [1, 10]$ whit p = 3, μ_1 = [2.9, 8.3, 5.7].

Between the mean vectors, two differences were considered between the μ_i´*s*. The small differences consist of differences between 10 and 20 % compared to μ_1. e.g. $\mu_2 = \mu_1$ x 1.1 (10 % difference to μ_1). The large differences include very high percentages between the differences between the mean vectors, ranging from 50 to 300 % compared to μ_1, e.g. $\mu_2 = \mu_1$ x 3 (300 % difference compared to μ_1).

Covariance Matrices (Σ´s)

For uncorrelated variables (Case 1), $\Sigma = \sigma^2 I_p$, considering σ^2 = 1 or 10. In the case of correlated variables (Case 2), the *genPositiveDefMat* function of the *clusterGeneration* library (Qiu and Joe, 2023) was used to generate positive definite random variance and covariance matrices. With this function, two matrices were generated: one with variances in the range [1, 2] (together with their respective covariances) and another with variances in the range [8, 12] to emulate the variances in the matrices studied in the case of uncorrelated variables, with the following conditions: number of treatments: t = 3,7; number of variables: p = 3,7; and number of replicates per treatment: r = 4,16.

The data were analyzed with MANOVA and the value of the approximation to the Pillai Trace statistic F (υ_{H}, υ_{E}) (Pillai and Samson, 1959) and the p - *value*. In cases of three treatments with three variables and four replicates, confidence intervals were obtained using Tukey's generalized conjecture (Seo and Fujikoshi, 1994). In other cases, this determination could not be made due to the limited degrees of freedom.

The data were then transformed using the proposed methodology and analyzed using ANOVA for the CRD model. In this case, the assumptions of normality and homoscedasticity were checked for compliance with the Shapiro-Wilk (SW) (Shapiro and Wilk, 1965) and Levene (L) (Levene, 1960) tests. If the transformed data did not meet any of the assumptions, the Box-Cox transformation (Box and Cox, 1964) was performed. If ANOVA rejects the null hypothesis of equal treatment effects, the comparison of means was performed using Tukey's test.

RESULTS AND DISCUSSION

The proposal to transform the vectors generated in a CRD into scalars and test the hypothesis of equal treatment effects with ANOVA performs satisfactorily in terms of power. There is a good performance of the test power with increasing sample size (Figures 1, 2, and 3), which is expected according to statistical theory (Casella, 2008; Hinkelmann and Kempthorne, 2005). As the number of variables (p) increases, the power of the test also increases, suggesting that the asymptotic result of convergence from the X_{ij} normal distribution works with medium sample sizes. Finally, the results show that, as the maximum differences between the vectors increase, the power of the test increases, which is also the expected behavior (Casella, 2008).

When there are four repetitions to achieve a power greater than 0.8, the difference between the mean vectors is required to be at least 40 %. For the cases of 8, 12, and 16 repetitions, this power is obtained when there is a 20 to 30 % difference between the mean vectors. It should be noted that this simulation study was much more extensive; however, only a small sample is presented to show the relevant aspects..

Comparison of MANOVA performance against the proposed methodology

The results of the performance comparison between the MANOVA and the methodology proposed in the test of the hypothesis of equal treatment effects are also satisfactory in the case of a CRD with multiple responses. The methodological approach in most cases detects smaller differences than the MANOVA (Table 2). In some cases, MANOVA cannot be used to analyze the data due to the limitations of the methodology (Table 3), because when r-1>p, the residual matrix W is not of full rank, and hence the test statistic $\Lambda^* = |W| \; / \; |B + W|$ used in MANOVA is not useful because $|W| = 0$ (Strang, 2006).

While the methodological proposal does not present such a problem, the multivariate case was transformed into the univariate case, and therefore the analysis in this case

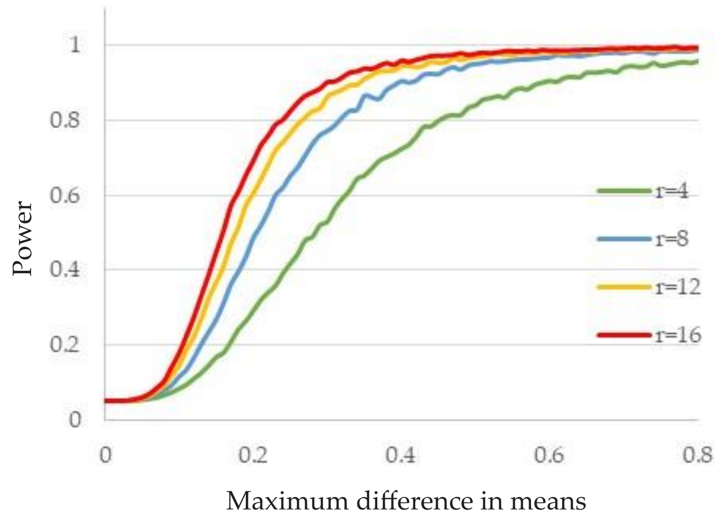

Figure 1. Estimated powers for $p = 3$ (number of variables) in $t = 3$ (number of treatments) with r replicates per treatment: $\mu_1 = [\mu_1, \mu_2, \mu_3]^t$, $\mu_1 \in [1, 10]$, $\mu_2 = (1 + escal\ [h]) \times \mu_1$, $\mu_3 = (1 + escal\ [h]) \times \mu_1$; scal=[0, 0.01, 0.02, …,0.8], for h=1, 2,…; $\Sigma = I_p$; number of Monte Carlo samples $B = 2000$; level of significance used: $\alpha = 0.05$.

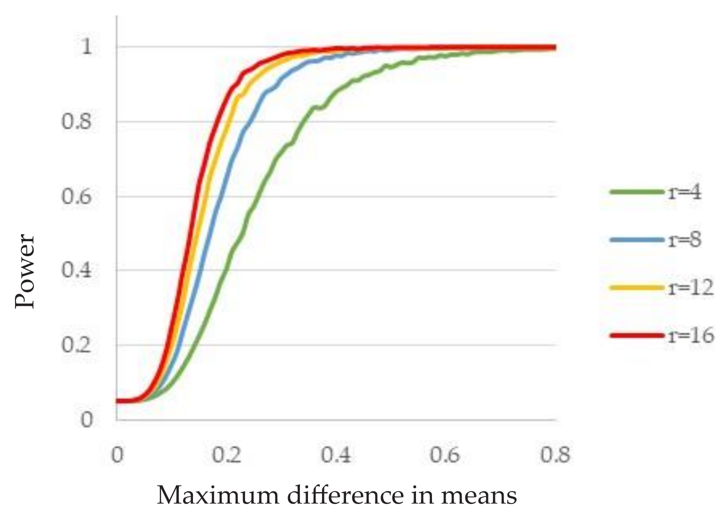

Figure 2. Estimated powers for $p = 5$ (number of variables) in $t = 3$ (number of treatments) with r replicates per treatment: $\mu_1 = [\mu_1, \mu_2, \mu_3, \mu_4, \mu_5]^t$, $\mu_1 \in [1, 10]$, $\mu_2 = (1 + escal\ [h]) \times \mu_1$, $\mu_3 = (1 + escal\ [h]) \times \mu_1$; scal=[0, 0.01, 0.02, …,0.8], for h=1, 2,…; $\Sigma = I_p$; number of Monte Carlo samples $B = 2000$; level of significance used: $\alpha = 0.05$.

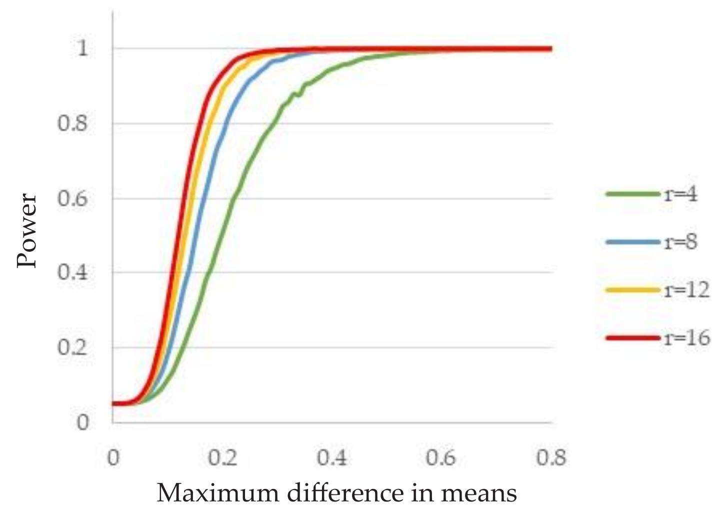

Figure 3. Estimated powers for p = 7 (number of variables) in t = 3 (number of treatments) with r replicates per treatment: $\mu_1 = [\mu_1, \mu_2, \mu_3, \mu_4, \mu_5, \mu_6, \mu_7]^t$, $\mu_1 \in [1, 10]$, $\mu_2 = (1 + escal\,[h]) \times \mu_1$, $\mu_3 = (1 + escal\,[h]) \times \mu_1$; scal=[0, 0.01, 0.02, …,0.8], for h=1, 2,…; $\Sigma = I_p$; number of Monte Carlo samples B = 2000; level of significance used: α = 0.05.

can be carried out. A satisfactory performance of the methodological approach can be observed when correlated observations are available (Table 4). When the maximum differences between the mean vectors are greater than 100 %, the MANOVA and the methodological approach reject the hypothesis of equal treatment effects, although the significance level is higher in the MANOVA. It was also observed that, in some cases, the transformed data did not meet the assumption of normality and generally met the assumption of homoscedasticity.

Often, the problem of non-compliance with the assumptions was solved with the Box-Cox transformation. No changes in the significance of the MANOVA and ANOVA are observed when changing the variance and covariance matrix. In all cases studied, Tukey's HSD methodology presented results in accordance with the simulation parameters. This research consisted of studying the parameters established in the methodology and generating results with simulation in 46 tables, although only a sample is presented here in order to show the relevant aspects.

The results of using the proposed methodology to test the hypothesis of equality of treatments with multiple responses generated by a CRD with correlated normal variables are satisfactory. This methodology could be applied to the case of other experimental designs after investigating their performance through simulation.

Table 2. Comparisons for case t = 3 (number of treatments), p = 3 (number of variables), r = 4 (number of replicates per treatment), and small differences between μ'^{s}_{i}.

μ	Σ	MANOVA $\sim F_{vH, vE}$	Tukey's generalised conjecture	Transformed data p - value		ANOVA $\sim F_{v1, v2}$	HSD Tukey
		Pr (> F)		SW	L	Pr (> F)	
μ_1 = [2.9, 8.3, 5.7] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	I_3	1.638 0.201	T1 vs. T2: [-10.8, 7.4] T1 vs. T3: [-12.5, 5.7] T2 vs. T3: [-10.8, 7.4]	0.0201	0.960	5 0.035	T3 662.025 a T2 519.346 ab T1 398.076 b
μ_1 = [90.3, 57.6, 82.3] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	I_3	2.663 0.055	T1 vs. T2: [-32.1, 13.8] T1 vs. T3: [-55.1, -36.8] T2 vs. T3: [- 32.1, 13.8]	0.0318	0.954	597.2 2.7×10^{-10}	T3 33 811.123 a T2 23 828.921 b T1 16 239.583 c
μ_1 = [2.9, 8.3, 5.7] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	10 * I_3	0.366 0.890	T1 vs. T2: [-30.6, 27.2] T1 vs. T3: [-32.3, 25.5] T2 vs. T3: [- 30.6, 27.2]	0.0654	0.909	0.705 0.519	No significant difference
μ_1 = [90.3, 57.6, 82.3] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	10 * I_3	2.633 0.057	T1 vs. T2: [-51.9, 5.9] T1 vs. T3: [-74.9, -17.0] T2 vs. T3: [- 51.9, 5.9]	0.0351	0.9517	61.29 5.73×10^{-6}	T3 32 397.475 a T2 22 749.481 b T1 15 437.039 c

Table 3. Comparisons for case t = 3 (number of treatment), p = 7 (number of variables), r = 4 (number of replicates per treatment), small differences between μ'^{s}_{i}.

μ	Σ	MANOVA $\sim F_{vH, vE}$	Transformed data p - value		ANOVA $\sim F_{v1, v2}$	HSD Tukey
		Pr (> F)	SW	L	Pr (> F)	
μ_1 = [2.9, 8.3, 5.7, 7.6, 1.3, 6.0, 9.5] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	I_7	Residuals have rank 3 < 7	0.056	0.971	28.14 0.0001	T3 411.386 a T2 345.129 b T1 284.782 c
μ_1 = [21.6, 5.9, 14.1, 8.1, 17.5, 23.7, 5.7] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	I_7	Residuals have rank 3 < 7	0.2872	0.9494	413.5 1.39×10^{-9}	T3 2332.021 a T2 1954.931 b T1 1611.166 c
μ_1 = [2.9, 8.3, 5.7, 7.6, 1.3, 6.0, 9.5] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	10 * I_7	Residuals have rank 3 < 7	0.112	0.973	2.111 0.177	No significant difference
μ_1 = [21.6, 5.9, 14.1, 8.1, 17.5, 23.7, 5.7] $\mu_2 = \mu_1 \times 1.1$ $\mu_3 = \mu_1 \times 1.2$	10 * I_7	Residuals have rank 3 < 7	0.050	0.905	32.34 7.78×10^{-5}	T3 2229.853 a T2 1866.041 b T1 1535.553 c

Table 4. Comparisons for case t = 3 (number of treatments), p = 3 (number of variables), r = 4 (number of replicates), large differences between μ'^{s}_{i}, correlated variables.

μ	Σ	MANOVA $\sim F_{vH, vE}$ $Pr (> F)$	Tukey's generalised conjecture	Transformed data *p - value* SW	L	ANOVA $\sim F_{v1, v2}$ $Pr (> F)$	HSD Tukey
μ_1 = [2.9, 8.3, 5.7] $\mu_2 = \mu_1 \times 3$ $\mu_3 = \mu_1 \times 2$	A	2.666 0.0548	T1 vs. T2: [-38.9, -28.6] T1 vs. T3: [-22.0, -11.7] T2 vs. T3: [11.1, 22.6]	0.498	0.295	183.4 5.09×10^{-8}	T2 1010.855 a T3 456.426 b T1 121.577 c
μ_1 = [90.3, 57.6, 82.3] $\mu_2 = \mu_1 \times 3$ $\mu_3 = \mu_1 \times 2$	A	2.667 0.055	T1 vs. T2: [-465.5, -455.2] T1 vs. T3: [-235.3, -225.0] T2 vs. T3: [224.4, 235.9]	0.439	0.542	654865 $< 2 \times 10^{-16}$	T2 164033.520 a T3 7286.500 b T1 18191.760 c
μ_1 = [2.9, 8.3, 5.7] $\mu_2 = \mu_1 \times 3$ $\mu_3 = \mu_1 \times 2$	B	2.659 0.055	T1 vs. T2: [-47.9, -19.6] T1 vs. T3: [-31.0, -2.7] T2 vs. T3: [2.7, 31.0]	0.731	0.245	166.9 7.71×10^{-8}	T2 1010.454 a T3 467.568 b T1 144.262 c
μ_1 = [90.3, 57.6, 82.3] $\mu_2 = \mu_1 \times 3$ $\mu_3 = \mu_1 \times 2$	B	2.667 0.055	T1 vs. T2: [-474.5, -446.2] T1 vs. T3: [-244.3, -216.0] T2 vs. T3: [214.4, 24.9]	0.331	0.451	32511 $<2 \times 10^{-6}$	T2 164116.560 a T3 72934.270 b T1 18242.260 c

$$A = \begin{pmatrix} 1.5 & -0.6 & -1.1 \\ -0.6 & 1.7 & 0.3 \\ -1.1 & 0.3 & 1.2 \end{pmatrix};\ B = \begin{pmatrix} 8.6 & 3.0 & -8.3 \\ 3.0 & 10.7 & 0.1 \\ -8.3 & 0.1 & 10.1 \end{pmatrix}$$

CONCLUSIONS

In this research, an alternative analysis was proposed to test the hypothesis of equality of effects between treatments and *post hoc* tests in the case of multiple responses. The simulation study shows that the performance of the proposal with small samples is satisfactory in terms of power and that it has advantages compared to MANOVA. Furthermore, the methodological approach allows for *post hoc* testing in the case of multiple responses in the completely randomized experimental design. The transformed data, from the proposed methodology, have problems holding the normality assumption when the number of variables (p) is relatively small, which is usually solved by the Box-Cox transformation.

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GROWTH AND ACCUMULATION OF PHENOLIC COMPOUNDS IN THYME (*Thymus vulgaris*) BASED ON THE BALANCE OF RED AND BLUE LED LIGHTS

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ABSTRACT

The new plant production methods that use artificial light to replace or complement sunlight have proven that changes in the wavelength of incidental light result in variations in growth, development and secondary metabolism of plants, depending on the genotype and other environmental conditions. However, these methods have been scarcely studied in medicinal and edible plants. The aim of this study was to determine the response of thyme plants (*Thymus vulgaris*) under different wavelengths. The plants were exposed to red light (660 nm), blue light (440 nm), white light and two proportions of red-blue for 16 hours a day at an intensity of 25 μmol m^{-2} s^{-1}. The treatments were isolated from sunlight and from each other. Red light was found to promote the formation of etiolated plants, with a low accumulation of chlorophyll, dry matter and phenolic compounds compared to the white light treatment. Blue light generated compact plants with a higher accumulation of chlorophyll and dry matter than red light, but similar to the white light treatment. In terms of phenolic compounds, accumulation was higher under the two latter treatments. The planting of thyme under a combination of blue-red light at a 3:1 ratio was found to result in a compact growth and to improve the accumulation of phenolic compounds.

Keywords: dry matter, secondary metabolism.

INTRODUCTION

In terms of plant food production, the past decades have been characterized by the search for methods that allow for the production of high amounts of high-quality, safe and environmentally friendly biomass in small spaces and with a high efficiency in the use of water, fertilizers and other inputs required for the production process. Vertical gardens, biofactories and the indoor plant production systems (IPPS) seem to be the production methods that best achieve these goals (Bures *et al*., 2018).

The lighting environment is one of the most carefully handled aspects of these production systems, since photosynthesis and photomorphogenesis depend mostly on the wavelength, intensity and duration of light exposure (Casierra-Posada and Peña-Olmos, 2015; Alrifai *et al*., 2019). LED lighting technology, due to its high electrical energy efficiency, low heat emission and high manipulation of the intensity and quality of the light emitted (Dutta-Gupta and Agarwal, 2017), has become the most widely used light source in these production systems, replacing or complementing sunlight (in some cases).

IPPSs are mainly used in the production of vegetables with a high economic value or of plants whose high market value is attributed to their useful phytochemical properties, such as medications or food. Reports show that the efficiency of an IPPS in in the production of biomass or secondary metabolites strongly depends on the genotype (Alrifai *et al*., 2019). In other words, the different species, and even different cultivars, respond in different ways when established in these systems, due to the effect of the quality of incidental light. This is a technical and scientific opportunity for research, which we decided to explore in this study.

Thyme (*Thymus vulgaris*), a species of the Lamiaceae family, is used in both cuisine and traditional medicine for its secondary metabolites (Hosseinzadeh *et al*., 2015). It is a highly branched plant, with a maximum height of 50 cm, rich in phenolic compounds, and its essential oil is mainly composed of the monoterpenes thymol and carvacrol, which give it its distinct aroma (Gimeno-Gasca, 2001).

In the IPPSs, research has focused mainly on the use of blue light (420–480 nm), red lights (620–700 nm), combinations of these, and white light, with other wavelengths being used in a secondary manner (Landi *et al*., 2020). In lamiaceae, research focuses on species with culinary uses. In basil (*Ocimum* sp.), blue light, in comparison with red and white lights, promotes the formation of more compact plants (Matysiak and Kowalski, 2019). Likewise, the use of red light has been reported to help improve the antioxidative capacity of the extracts of this plant (Taulavuori *et al*., 2016). In Mexican mint (*Plectranthus amboinicus*), Noguchi and Amaki (2016) discovered that the wavelength of blue light (100 μmol m^{-2} s^{-1}) promotes apical dominance and the formation of compact plants, in comparison with treatments with the wavelengths of red and green lights, which promote less compact plants, and with greater lateral growth.

The use of low intensities of LED lights (< 50 μmol m^{-2} s^{-1}) is not common in IPPSs, although it is well-known that most plant photoreceptors respond to light intensities below 1 μmol m^{-2} s^{-1} (Paradiso and Proleitti, 2022). In this sense, thyme has been reported to resist low light conditions (Murillo-Amador *et al.* 2013; Tabbert *et al.* 2021), therefore investigating the responses of these plants to conditions of low light intensity is a great opportunity. This study applied LED light with wavelengths corresponding to the colors white, red, blue, red-blue 1:3 and red-blue 3:1 at 25 μmol m^{-2} s^{-1} on young thyme plants, with the main purpose of evaluating the effect on the growth and accumulation of phenolic compounds and flavonoids. The intention of this is to contribute to the knowledge on the effect of applying different wavelengths at low intensities on this species in order to have a solid basis for its plantation in IPPS, as well as to contribute to the scientific knowledge in this area of plant physiology.

MATERIALS AND METHODS

The experiment was carried out between the months of April and June, 2021, in a greenhouse of the postgraduate department of horticulture at the Chapingo Autonomous University. Thyme seedlings were used, obtained from seeds (Vita®) which were germinated under greenhouse conditions, with a substrate composed of 70% peat and 30% perlite. During the first two weeks after germination, tap water was used to irrigate the plants. Fourteen days after germination, the plantlets were established in pots measuring 4 inches in diameter, with a substrate composed of 50% peat, 48% perlite and 2% vermicompost. Starting on that day and until the end of the experiment, 100mg L^{-1} of Multipurpose Ultrasol® fertilizer was applied once a week and irrigating with tap water every 2 days. Plants were kept under conditions of natural light for 28 days. On days 10 and 24, they sprouted to promote branching and the homogenization of the aerial section. They were then moved to conditions of entirely controlled light, where they remained for 35 days until the growth and secondary metabolism analyses.

As a light source for the colors blue and red and their combinations, RGB 5050 (Weluvfit®) LED strips were used, each with 30 modules per meter (5 m), mounted on wooden boards measuring 15x40 cm, and which help modify the light spectrum between 400 and 700 nm, adjusted to the requirements of each treatment (Table 1). As a source of white light, white 3528 (Tunix®) LED strips were used, each with 60 modules per meter. In both cases, the efficiency of the strips did not change in the duration of the experiment. The height of the boards was adjusted for the light to fall on the plants, at an intensity of 25 $\mu mol\ m^{-2}\ s^{-1}$ between 14 and 21 cm (Table 1), using an Apogee® QMSW-SS radiometer. The photoperiod was 16 hours, starting at 6:00 h and ending at 22 h. The treatments were established in boxes 80x40x80 cm in length, width and height, respectively, with a white interior in order for the light to be

Table 1. Treatments used and height of the light plates in relation to the plants.

Treatment	Color	Height of light plate (cm)
1	White light	21[¶]
2	Red light(660 nm)[†]	12
3	Blue light (440 nm)	16
4	75% red light (660 nm) and 25% blue light (440 nm).	14
5	75% blue light (440 nm) and 25% red light (660 nm).	14

[†]Wavelength (nm) pointed out by the supplier. [¶]Measured from the highest part of the plant.

reflected and distributed homogenously. Air circulation was provided by a 4-inch, 12 V fan placed on the rear side of the box, which was turned on for 10 min every hour between 6:00–11:00 and 18:00–22:00, and for 15 min between 12:00 and 17:00. Inside the boxes, the board with the LEDs for each treatment were placed on the top. The experimental unit consisted of one thyme plant. Five treatments were established with 10 repetitions each (Table 1). The experimental design was completely randomized. Inside each box, plants were rotated every day in order to reduce error due to the incidence of light on the repetitions in each treatment.

Thirty-five days after the treatments began, plant heights and the number of branches (tertiary, quaternary and subsequent) were recorded. The relative chlorophyll index was calculated using a SPAD unit meter by KONICA MINOLTA®, model SPAD-502Plus; each measurement -one repetition- came from the average of 10 random takes across the length and width of the plant. The fresh and dry weights of the aerial section and of 30 specific leaves were recorded using a METTLER® model AJ150L analytic scale; for drying, a Márquez® stove was used, at 60 °C until constant weight. Given the difficulty of separating all the leaves of the plant, as well as their tiny size and the speed at which they dehydrate, a sample of 30 leaves was taken from the low part of the branches formed during the light treatments and it was measured using a LI-COR®, model LI-3100 foliar area integrator. The specific weight of the leaves was calculated by dividing the dry weight of the leaf by the foliar area.

For the extraction of phenolic compounds, 2 g of fresh and ground plant material was used per repetition. It was then placed into test tubes and 10 ml of 80% methanol was added. The tubes were covered and taken to a bath with ultrasound in a cycle, 10 min on, 5 min off and another 10 min on. Finally, the plant material was separated from the extract by centrifuging at 1000 g for 5 min. To quantify the total phenolic compounds (T.P.C.) from the supernatant, a 20 μL aliquot was taken, which was incubated with 480 μL of water, 25 μL of a Folin-Ciocalteau reagent solution 1:1 and 975 μL of a 2.5% sodium carbonate solution for an hour at room temperature. The absorbance of the colored product was measured at 740 nm in a spectrophotometer against a white containing no gallic acid nor extract. The concentration value the T.P.C. was calculated from a standard gallic acid curve (1–20 mg L^{-1}, coefficient of correlation $R^2 = 0.9905$).

To quantify total flavonoids (T.F.), a 20 μL aliquot was taken from the extract used in the quantification of T.P.C. and incubated for 40 min at room temperature with 980 μL of methanol at 80 %, 2000 μL of potassium acetate 1 M and 2000 μL of 10% aluminum chloride. Absorbance was measured at 415 nm with a spectrophotometer against a white without any quercetin nor extracts of the sample. The value of the concentration of the T.F. was calculated from a standard quercetin curve (1.25–10 mg L^{-1}, coefficient of correlation $R^2 = 0.96$).

An analysis of variance (ANOVA) was performed by each of the variables considered, with a significance level of 0.05; in case it resulted significant, Tukey's multiple means comparison test ($p \leq 0.05$) was carried out. To compare the different treatments against the white light treatment, Dunnett's test ($p \leq 0.05$) was carried out. The SAS statistical program, version 9.0, was used.

RESULTS AND DISCUSSION

The chances promoted by the treatments were statistically significant ($p \leq 0.05$) for height, number of branches, and the compacting of thyme plants. Height recorded the highest values in treatment four with significant differences with all the other treatments (Table 2). This treatment also generated the greatest fresh weight, but without statistical differences for this variable, with treatments one and five. These results can be explained considering that the growth and development of a plant is influenced, among many other factors, by the growth and development of phytochromes (stimulated by red, distant red and blue lights) and of cryptochromes (stimulated by blue and green lights) and the balance between them (Stutte, 2009; Casal, 2013).

Table 2. Average values for the variables of height, number of branches, compacting, fresh weight and dry weight of thyme plants (*Thymus vulgaris*), treated with different colored lights for 35 days (n = 7).

Treatment	Height (cm)	Number of branches	Compacting (g m^{-2})	Fresh weight (g)	Dry weight (g)
1	19.10 b[†]	31.50 b	1.90 a	3.57 ab	0.36 a
2	19.47 b	32.75 ab	1.10 b	2.57 b	0.21 c
3	16.40 b	36.50 ab	1.67 a	2.55 b	0.27 bc
4	28.02 a	43.25 a	0.94 b	4.35 a	0.25 bc
5	18.97 b	36.00 ab	1.69 a	3.20 ab	0.32 ab
DMSH[¶]	7.17	11.43	0.25	1.19	0.073
C.V.[§]	16.1	14.54	7.81	16.84	11.73

[†]Measurements with the same letters in each column are not different (Tukey, $p \leq 0.05$). [¶]HLSD: honest least significant difference; C.V.[§]: coefficient of variation.

White light promotes a similar balance to sunlight, whereas monochromatic treatments with red or blue light induce a clear imbalance, which leads to a reduction in growth (Landi *et al*., 2020). In the case of red-blue combinations, the proportion determines a greater or reduced growth (Hernández *et al*., 2016). The results show that, in thyme, a greater proportion of red light promotes an increase in growth; in addition, they partially coincide with those reported for tomato plants, where a reduction in the red-blue proportion reduces growth, with its peak in the treatment with red light (Hernández *et al*., 2016).

The dry weight was greater in treatments one and five, and lower in the remaining treatments. A greater accumulation of dry weight in polychromatic treatments and under a high ratio of red-blue light has also been observed in *Mentha longifolia* (Sabzalian *et al*., 2014), *Oncidium* 'Gower Ramsey' (Chung *et al*., 2010) and cucumber

(Cucumis sativus L*)* (Miao *et al*., 2016). This effect has been related to the photosynthetic process, since it has been proven that white light itself and blue light (compared to red light) promote a higher proportion of a/b chlorophyll a/b (Hamdani *et al*., 2019), the adequate functioning of stomata (Lanoue *et al*., 2018), an adequate distribution of chloroplasts (Su *et al*., 2014), a greater abundance of the enzyme Rubisco (Landi *et al*., 2020) and a high rate of net assimilation (Lanoue *et al*., 2018).

Compacting is the relation between dry weight and height. It represents a quality parameter in plants that, after being harvested, are dehydrated for their conservation and later use, as in the case of thyme. The results show that, although there were differences between treatments, only white light maintained a maximum value, despite not being statistically different to T3 and T5 (Table 2).

Another parameter with a desirable increase in thyme is branching. Our results show significant differences ($p \leq 0.05$) between the treatment with white light (T1) and the R-B 3:1 (T4) (Table 3), the latter displaying the highest mean. Greater branching is related to a greater sprouting of auxiliary buds, promoted by a change in the balance of auxins and cytokinins, which tends towards the latter (Casal, 2013). This tendency in T4 may two explanations: 1) the greater height of plants under this treatment reduces the concentration of auxins (basipetal movement from the apex) in the lowest buds of the branches, while the concentrations of cytokinins (acropetal movement from the root) is not altered (given that the buds are very close to the roots), which promotes their sprouting; and 2) the hormonal imbalance is an effect of the quality of the incidental light. The precedents on the effect of the quality of light on the hormonal balance that promotes the sprouting of buds are very scarce.

The effect of the quality of incidental light on thyme plant leaves resulted in statistically significant lights for all the variables evaluated ($p \leq 0.05$) (Table 4). The highest value

Table 3. Comparison of the averages of the response variables in thyme plants (*Thymus vulgaris*) treated with different colored lights in regard to white light, taken 35 days after the beginning of treatments (n = 7).

Variable	White	T2	T3	T4	T5
Height (cm)	19.1	=[†]	=	31.83 %	=
Number of branches	31.5	=	=	27.16 %	=
Compacting (g m^{-1})	1.90	-42.10 %	=	-50.52 %	=
Fresh wieght (g)	1.57	=	=	=	=
Dry weight (g)	0.36	-41.66 %	-25 %	-30.55 %	=
Leaf area(cm^2)	7.42	=	=	=	=
Estimated chlorophyll (SPAD)	34.55	=	=	=	=
Specific leaf weight (mg cm^{-2})	2.72	-25.73 %	=	=	-13.02 %

[†]The symbol "=" indicates that there are no significant differences (Dunnett, $p \leq 0.05$) between treatments for the indicated variable. Percentages indicate the magnitude of positive or negative variation in regard to the treatment with white light.

Table 4. Comparison of the leaf variables of fresh weight, dry weight, SPAD, leaf area and specific weight in response to the treatments with light on thyme plants (Thymus vulgaris) taken 35 days after the experiment began (n = 7).

Treatment	Fresh leaf weight (mg)	Dry leaf weight (mg)	Estimated chlorophyll (SPAD)	Leaf area (cm^2)	Specific weight (mg cm^{-2})
1	215.02 a[†]	20.20 a	34.55 ab	7.42 ab	2.72 ab
2	160.57 e	16.27 d	33.20 b	8.09 a	2.02 c
3	204.55 b	19.47 b	37.52 a	6.70 b	2.91 a
4	187.40 c	18.72 c	33.95 ab	6.54 b	2.87 a
5	167.07 d	16.05 d	37.25 ab	6.76 b	2.37 bc
DMSH[¶]	5.29	0.55	4.16	1.03	0.40
C.V.[§]	1.29	1.40	5.40	6.66	7.09

[†]Means followed by the same letter in each column are not different (Tukey, $p \leq 0.05$). [¶]HLSD: honest least significant difference; [§]C.V.: coefficient of variation.

for fresh weight was obtained in T1 and the lowest, in T2. In the case of dry weight, the highest value was observed in T1 and the lowest, in T2 and T5 (Table 4). A reduction in these variables due to red light was observed in chili pepper plants *(Capsicum annuum* L.*) by* Gangadhar *et al.,* (2012), who argued that the highest dry weight observed in the polychromatic treatments (red-blue, white) against the monochromatic ones was due to the fact that blue and red lights together, in the polychromatic treatments, increase the efficiency of the photosynthetic process, given that it is these precise wavelengths that absorb chlorophyll. However, the results from this study do not coincide with those observed in other Lamiaceae such as wild mint (*Mentha arvensis* L.) (Nishioka *et al.,* 2008) and perilla (*Perilla frutescens* L. Btitt) (Nishimura *et al.*, 2009), in which red light increased the fresh and dry weights of leaves.

The highest foliar area was obtained in the treatment with red light (T2), without any differences with white light (T1), while the remaining treatments displayed a significantly lower foliar area ($p \leq 0.05$) (Table 4). In perilla (Nishimura *et al.*, 2009) and wild mint (Nishioka *et al.*, 2008), an increase had already been reported in the foliar area under red light, in comparison with other light colors. Several studies have attributed foliar expansion promoted by red light to the influence the latter has on the calcium and potassium canals in the epidermal cells, which allow for a flow of ions which, via an osmotic process, promotes growth (Volkenburgh, 1999).

The specific weight of leaves has been used as an estimator of photosynthesis, of the interception and absorption of light, as well as an indicator of the accumulation of carbohydrates in these organs (González-Pérez *et al.*, 2018). In this study, T2 and T5 has a lower specific leaf weight (Table 4), in comparison with the other treatments. In the case of T2, the foliar area and dry weight are greater, whereas the content of chlorophyll is equal to T5, which led to the reduction of the specific weight of leaves related to a higher foliar expansion without an increase in photosynthesis.

The treatments applied generated changes in total phenols (Figure 1). The blue light (T3) and the combinations blue-red (T4 and T5) generated a higher concentration of phenols than in T1 and T2. For the flavonoids, the highest concentrations were recorded in T1 and T5 (Figure 1). In contrast to white light, the treatments with blue lights and R-B 25:75 were observed to increase the concentration of phenolic compounds (Table 4). In the case of the flavonoids, the red light reduced its concentration in regard to white light, whereas the blue light increased it (Table 5).

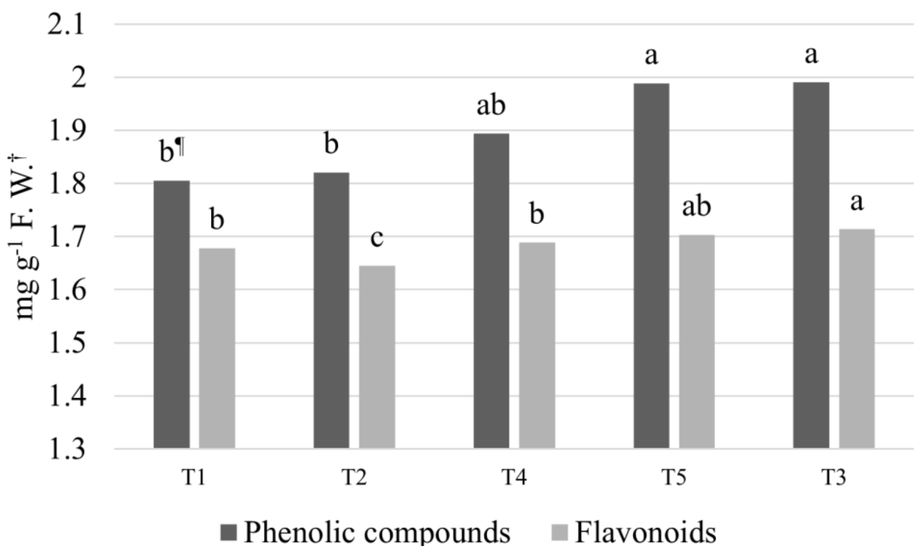

Figure 1. Effect of incidental light on the content of phenolic compound in thyme. †P. F.: dry weight. ¶Columns of the same variable with the same letter are not statistically different (Tukey, $p \leq 0.05$).

Table 5. Contrast of different light colors regarding white light on the concentration of total phenols and flavonoids in thyme plants (*Thymus vulgaris*) after 35 days of treatment.

Variable	T1	T2	T3	T4	T5
[Phenolic compounds] (mg g^{-1} P. F.†)	1.80	=¶	+10.26 %	=	+10.15 %
[Flavonoids] (mg g^{-1} P. F.)	1.67	-1.96 %	+2.12 %	=	=

†Fresh weight. ¶The symbol "=" indicates that there are no statistical differences (Dunnett, $p \leq 0.05$) between white light (T1) and the additional treatments for the variable indicated. Percentages indicate the magnitude of the positive or negative variation regarding the treatment with white light.

The biosynthetic pathways of the phenolic compounds are easily altered by the light conditions in which plants are found (Alrifai *et al*., 2019; Landi *et al*., 2020). Many reports coincide in that the blue light increases the biosynthesis and concentration of phenolic compounds, whereas red light produces the opposite effect in crops such as lettuce (*Lactuca sativa*) (Johkan *et al*., 2010), strawberry (*Fragaria vesca*) (Choi *et al*., 2015) and basil (*Ocimum basilicum*) (Taulavuori *et al*., 2016).

The role of blue light in the biosynthetic pathway of phenolic compounds is related to key enzymes in this pathway, such as PAL (phenylalanine ammonia-lyase), the activity of which increases under different artificial light qualities (Kim *et al*., 2015). Likewise, there have reports of an increase in the expression of the genes that codify the enzymes C_4H (cinnamic 4-hidroxylase), CHI (chalcone isomerase), FLSII (flavonol synthase II), ANS (anthocyanidin synthase) (Thwe *et al*., 2014), F3´H (flavonoid 3´ hydroxylase) and FLS (flavonol synthase), under blue light in contrast with white and red light (Kim *et al*., 2015), which participate in different biosynthesis pathways of phenolic compounds.

Regarding flavonoids, there have also been reports of a similar behavior to that of phenolic compounds in general, in the sense that blue light increases its synthesis (Taulavuori *et al*., 2016). Liu *et al*. (2018) found that the increase in the synthesis of flavonoids under blue and green light (in comparison to white and red light) is correlated to a greater expression of the genes that codify enzymes PAL, 4CL and CHS. These enzymes participate in the synthesis pathway of this group of polyphenols, which may support the explanation of the behavior described.

CONCLUSIONS

Treating thyme plants with a 75% blue – 25% red light combination produces plants with an adequate growth, since it favors the compacting accumulation of dry matter and a higher concentration of phenolic compounds.

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MORPHOLOGICAL AND BIOCHEMICAL ANALYSES OF *Agave salmiana* VARIETIES

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ABSTRACT

The pulque maguey is a perennial plant that provides several environmental benefits. Although its plantation has been drastically reduced, its primary economic and cultural use is the production of fermented, low-cost beverages. In this study, morphological and chemical analyses were conducted, which contribute to the knowledge that allows for the extraction of high-value products from pulque maguey biomass. The *Agave salmiana* varieties studied were Ayoteco (Ayo), Púa Larga (PL), Manso (Man), Chalqueño (Chal), Blanco Cenizo (BC), and Sha'mini (Sha), as well as a "Verde" variety known as Cosmimaco (VC). The data was analyzed using the Shapiro-Wilk test ($\alpha = 0.05$), followed by multiple means comparison using the Tukey procedure ($\alpha = 0.05$), the Kruskal-Wallis non-parametric test, and the Dunn-Bonferroni test ($\alpha = 0.05$). In order to differentiate the agave varieties by their chemical compositions, a canonical discriminant analysis (CDA) was applied. The lipid percentage (1.1–1.7 %) and the cellulose content (61–71 %) were higher than those reported for other *Agave* species. The BC variety contains a high concentration of cellulose and the least amount of lignin, making it a viable option for the energy industry. The CDA identified two discriminant functions that explained 95 % of the variance between the *A. salmiana* species in this study: ash content and lignin content. The results indicate that the amount of cellulose in pulque maguey leaves is an option to obtain products with a higher commercial value.

Keywords: Agavaceae, canonical discriminant analysis, pulque maguey.

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INTRODUCTION

The pulque maguey is a member of the *Agave* genus, which is native to the Americas. Mexico is home to 160 of its 210 species, 119 of which are endemic to the country. There are over 30 reported species of pulque maguey, from which various products can be obtained, such as *aguamiel*, which is fermented to produce pulque (Figueredo-Urbina *et al*., 2021). In this process, the leaves are left as waste on the field, though they can be used in cuisine, as forage, or to produce fiber or bioethanol (Narváez-Suárez *et al*., 2016). The leaves have also been studied for the extraction of secondary metabolites, such as steroidal saponins with pharmacological importance (Sidana *et al*., 2016) and phenols with a wide range of biological applications (Almaraz-Abarca *et al*., 2013).

The most widely used species for the production of pulque are *A. salmiana*, *A. mapisaga*, and *A. atrovirens* (Alfaro-Rojas *et al*., 2007). Depending on the variety, it takes anywhere between 8 and 14 years to produce *aguamiel*. This is why agave crops are disappearing, as they are being replaced by annual plantations such as barley. Furthermore, among other causes are the low cost of pulque and the reduction in its consumption (Ramírez-Manzano *et al*., 2020). Following the productive stage of the maguey, the leaves become agro-industrial waste with a high cellulose content. This biomass could be used in various processes, such as the production of second-generation bioethanol, because it does not compete with the food production areas (Yang *et al*., 2015; Díaz-Blanco *et al*., 2018; Jones *et al*., 2020; Yan *et al*., 2020), as a substrate in the production of fungi (Velázquez-de Lucio *et al*., 2022), and for the production of paper (Jiménez-Muñoz *et al*., 2016) and cellulose nanoparticles (Ponce-Reyes *et al*., 2014).

The name of the pulque magueys varies depending on the area (Ramsay, 2004), so the goal of this study is to characterize the morphology and chemical composition of seven varieties of pulque maguey, thereby contributing to technical knowledge that allows for the development of options for the use of the biomass to produce various commercially viable products, which encourages their plantation.

MATERIALS AND METHODS

Area of study and collection

In November and December 2016, maguey leaves were collected during the aguamiel production stage. In the "San Isidro" ranch, located in the municipality of Nanacamilpa, in the state of Tlaxcala, Mexico (19° 28′ 53′′ N, 98° 33′ 55.2′′ W; altitude 2800 m), the varieties Ayoteco (Ayo), Púa Larga (PL), Manso (Man), and Chalqueño (Chal) were gathered. In the town of Ayotla, located in the municipal area of Zacatlán, Puebla, Mexico (19° 55′ 9.84″ N, 98° 02′ 16.08″ W; altitude 2540 m), Blanco Cenizo (BC) and Verde/Cosmimaco (VC) were gathered. The Sha'mini (Sha) variety was gathered in the town of El Saucillo, in the municipality of Huichapan, Hidalgo, Mexico (20° 19′ 1.4″ N, 99° 42′ 25.2″ W; altitude 2170 m).

At the time of sampling, the morphological measurements of each maguey were taken in triplicate: height, length, and width of the leaves, distance between the teeth, and dimension of the apical spine in mature specimens (aged 8 to 14 years) using a measuring tape (Truper, Mexico). Three leaves (high, medium, and low parts of the plant) were taken from three magueys of each variety. The material was transported in bags, labeled with the relevant data, and refrigerated for later analysis.

Chemical determination

Fragments were cut from various parts of the fresh stalks, and composite mixtures of each variety were prepared in triplicate for humidity determination using a thermobalance (Probacsa, Mexico). The stalks were cut into pieces by variety and the sap extracted with an EX-S industrial juice extractor (International, Mexico). The agave fiber was dried in a Baxter brand oven at 65 °C for 72 h before being transferred to the National Institute of Forestry, Agricultural and Livestock Research (INIFAP) where it was ground in a Thomas-Wiley blade mill (model No. 4, Thomas Scientific, Swedesboro, NJ, USA) and sieved with a T-40 mesh size (420 μm), leaving the material of each variety dry, ground, sieved and homogenized for subsequent determinations. The ash content in the agave samples was determined by weight difference after calcining the plant material for 4 h in a muffle at 500 °C using method 923.03 (AOAC, 2005). Nitrogen levels for each variety were determined using a Kjeldahl DEK-1 micro distiller (Sev-Prendo, Mexico) in accordance with AOAC method 2001.11 (AOAC, 2005). Protein content was estimated using the following equation:

$$\textit{Crude protein}\ (\%) = (\%\ \textit{N Kjeldahl})\ (F)$$

where % *N Kjeldahl* denotes the percentage of nitrogen determined by *Kjeldahl*, and *F* is the base protein factor of 6.25.

Lipid extraction was carried out in a Soxhlet system with hexane at 60 °C for 6 h. Extracts from agave varieties were eliminated sequentially for lignin determination in accordance with the TAPPI-204 standard (TAPPI, 2007), with an ethanol-benzene mixture (1:2) in the first extraction, ethanol in the second, and distilled water in the third. After the sample was free of extracts, the amount of insoluble lignin was determined using the T222 method, which used 72 % sulfuric acid to hydrolyze and solubilize the carbohydrates in the sample, followed by filtering, drying, and weighing the insoluble lignin. The percentage calculation was done using the following formula:

$$\%\ \textit{Lignin} = (A)\ 100/W$$

where A is the weight of lignin (g) and W is the dry weight of the sample (g).

Statistical analysis

A one-factor analysis of variance (ANOVA) was performed on all variables, considering the general linear model $y_{ij} = \mu + \tau_i + \in_{ij}$, where yij is the variable response, μ is the general mean, τ_i is the effect of the i-th factor, and $\in_{ij}$ is the random error. To ensure the model's residual values were normal, the Shapiro-Wilk test ($\alpha = 0.05$) was performed using SAS version 9.2 (SAS, 2000). A multiple comparison of means was performed using the Tukey parametric procedure ($\alpha = 0.05$) or the non-parametric Kruskal-Wallis test, in conjunction with the Dunn-Bonferroni test ($\alpha = 0.05$) (Corder and Foreman, 2014). The SAS DISCRIM procedure was used to perform a canonical discriminant analysis (CDA) on agave varieties to differentiate them based on their biochemical composition.

RESULTS AND DISCUSSION

The normality test of the residual values using the Shapiro-Wilk method revealed that the probabilities of the value of the statistic (W) for the variables leaf length and width, distance between teeth, and ash content of the agave varieties are significant ($p \leq 0.05$) (Table 1), while the rest of the variables have a value of W greater than 0.05, indicating a normal trend. Most variables measured between magueys differ, except for moisture, proteins, and lipids (F test, $p \geq 0.05$).

Table 1. Results of the Shapiro-Wilk test, F test, and Kruskal-Wallis normality test for the analysis of the morphological and biochemical variables in pulque maguey varieties of the Agave salmiana species.

Variable	Shapiro-Wilk test		F test		Kruskal-Wallis test	
	Statistic (W)	*p* value (Pr < W)	Statistic (F)	*p* value (Pr > F)	Statistic (W)	*p* value (Pr < W)
Height (m)	0.9555	0.4303	24.45	< 0.0001	–	–
Apical spine length (cm)	0.9643	0.6062	5.01	0.0062	–	–
Leaf length (m)	0.9033	0.0001	–	–	39.37	< 0.0001
Leaf width (cm)	0.9589	0.0342		–	29.8	< 0.0001
Distance between teeth (cm)	0.9882	0.0371	–	–	78.61	< 0.0001
Humidity (%)	0.9373	0.1925	1.7	0.1926	–	–
Ash (%)	0.8661	0.0081	—	–	19.08	0.004
Protein (%)	0.9892	0.9963	1.57	0.2276	–	–
Lipids (%)	0.9170	0.0755	2.7	0.059	–	–
Cellulose (%)	0.9476	0.3069	4.89	0.0068	–	–
Lignin (%)	0.9530	0.3868	18.22	< 0.0001	–	–

The F test revealed significant differences ($p \leq 0.05$) in the height of the pulque magueys (Table 1). In a study of 62 varieties of the Salmianae section, Mora-López *et al*. (2011) mention that *A. salmiana* var. *salmiana*, to which Ayo, Man, Chal, PL, BC, and Sha belong, showed the widest morphological variability due to the degree of domestication of the plants, where the height of the maguey and the mechanical protection structures mainly change, so the varieties grown in crops (*magueyales*) are larger. In this study, only PL is grown at a lower height, with no significant difference from BC, the largest of the varieties sampled on the edges of land divisions (boundaries), alongside VC and Sha (Table 2).

Table 2. Comparison of the means of morphological variables among the pulque maguey varieties of the *Agave salmiana* species studied.

Maguey	Height (m)[1]	Length of the AS (cm)	Leaf length (m)	Leaf length (Ranges)[2]	Leaf width (cm)	Leaf width (Ranges)[2]	Distance between teeth (cm)	Distance between teeth (Ranges)[2]	Description of the AS
Ayo	4.30 a†	6.00 ab	2.36	59.00 a	29.22	28.44 a	4.078	104.85 b	Tg¶
BC	2.77 bc	7.47 a	1.47	20.83 c	32.56	47.17 a	4.014	100.89 b	Tg
Chal	2.87 b	5.17 b	1.53	30.72 cb	31.11	39.00 a	6.461	204.32 a	Tr§
Man	2.93 b	6.37 ab	1.46	18.94 c	29.44	29.33 a	4.172	106.69 b	Cgᵇ
PL	2.83 bc	6.17 ab	1.64	45.17 ab	31.44	41.17 a	3.806	91.35 b	Cg
Sha	2.13 c	4.17 b	1.57	29.83 cb	23.44	5.33	4.094	103.60 b	Cg
VC	2.13 c	6.00 ab	1.44	19.50 c	29.89	33.56 a	5.586	173.81 a	Cg

Ayo: Ayoteco; BC: Blanco Cenizo; Chal: Chalqueño; Man: Manso; PL: Púa Larga; Sha: Sha'mini; VC: Verde Cosmimaco; AS: apical spine. [1]Tukey test (α = 0.05); [2]Dunn-Bonferroni test (α = 0.05). †Values followed by the same letters are not significantly different to each other. ¶tubular gray; §tubular red; ᵇconical gray.

The apical spine is a structural component of mechanical protection (Figure 1). Magueys with higher levels of domestication have a shorter apical spine (Colunga-Garciamarin and May-Pat, 1997). The BC variety collected on the borders has the largest tubular-shaped apical spine; however, Sha, one of the boundary varieties with the shortest and most conical spine (Table 2), does not meet this characteristic. The Chal maguey's thorn is red, which makes it stand out. The VC variety resembled Chal by having short leaves with the greatest distance between teeth.

In terms of chemical composition, the F test reveals no significant difference ($p > 0.05$) in moisture, protein, and lipid content between magueys (Table 1). Given that the varieties in this study are from the *A. salmiana* species, Ayo is molecularly more related to the *A. mapisaga* species (Trejo *et al*., 2020), as are the "Verde" varieties (Alfaro-Rojas *et al*., 2007). The lipid fraction (1.14–1.63 %) exceeds that of *A. sisalana* (Gutiérrez *et al*., 2008), *A. tequilana*, *A. angustifolia*, and *A. lechuguilla* (0.5–1 %) (Jiménez-Muñoz *et al*., 2016). The lipid fraction contains sterols, fatty acids, fatty alcohols, terpenes, long-

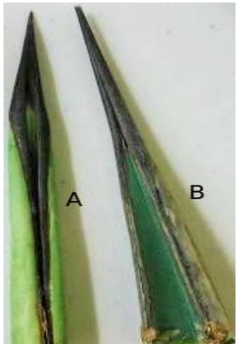

Figure 1. Type of apical spine in the analyzed pulque maguey varieties of the *Agave salmiana* species. A: tubular; B: conical.

chain alcohols, alkanes, ferulic acid esters, hydrocarbon steroids, monoglycerides, aldehydes, and sterol esters.

The cellulose and lignin contents vary significantly ($p \leq 0.05$). The VC variety has the most cellulose (Table 3). In bioenergy research, the most important materials are those with a high cellulose content and a low lignin content. Maguey has been reported to contain 40–80 % cellulose and 16–20 % lignin (Iñiguez-Covarrubias *et al*., 2001; Ponce-Reyes *et al*., 2014). Other studies report lignin percentages ranging from 5.3 to 9.75 % for *A. americana* (Li *et al*., 2014; Jones *et al*., 2020) and 8.4 to 11.9 % for *A. salmiana* (Li *et al*., 2014; de Dios-Naranjo *et al*., 2016). This study's cellulose content is higher than that reported by Yang *et al*. (2015) and Yan *et al*. (2020) for *A. tequilana* (26–26.5 %), and by Díaz-Blanco *et al*. (2018) for *A. lechuguilla* (20.18 %). In contrast, Iñiguez-Covarrubias *et al*. (2001) found 68 % cellulose, similar to the Ayo and VC varieties.

Table 3. Biochemical variables comparison of the Agave salmiana pulque maguey species studied.

Variety	Humidity (%)[1]	Ash (%)	Ash (Ranges)[2]	Protein (%)[1]	Lipids (%)[1]	Cellulose (%)[1]	Lignin (%)[1]
Ayo	87.03 a*	9.18	20.00 a	4.90 a	1.42 a	66.08 ab	16.87 c
BC	87.04 a	4.07	2.00 f	4.30 a	1.14 a	64.50 ab	13.54 d
Chal	85.39 a	5.23	7.33 ed	3.44 a	1.14 a	64.02 b	18.74 cb
Man	86.13 a	5.64	11.00 cd	3.91 a	1.26 a	62.46 b	16.74 c
PL	85.57 a	7.15	15.00 cb	3.97 a	1.37 a	61.02 b	20.05 ab
Sha	83.47 a	7.11	16.00 ab	3.58 a	1.57 a	63.56 b	17.98 cb
VC	85.37 a	4.77	5.67 ef	3.80 a	1.63 a	71.35 a	21.95 a

BC: Blanco Cenizo; Chal: Chalqueño; Man: Manso; PL: Púa Larga; Sha: Sha'mini; VC: Verde Cosmimaco. [1]Tukey test (α = 0.05); [2]Dunn-Bonferroni test (α = 0.05). *Values followed by the same letters are not significantly different to each other.

The biomass produced by maguey pulque trees can be converted to bioethanol, particularly the BC variety. Although it contains less cellulose, this is compensated by the lower amount of lignin that must be degraded for sugar utilization. Furthermore, in a recent study (Velázquez-de Lucio *et al*., 2022), *A. salmiana* was used as a substrate for the production of the *Pleurotus djamor* fungus with a protein percentage of 4.8 (similar to Ayo), which, despite being treated with urea, was a good alternative due to the chemical composition of maguey bagasse.

The ash content differs between magueys in this study ($p \leq 0.05$). The values of 4.42–9.7 % are similar to those reported for *A. americana* (Jones *et al*., 2020), *A. lechuguilla* (Díaz-Blanco *et al*., 2018), and *A. tequilana* (Yang *et al*., 2015; Yan *et al*., 2020). Chemical composition differences are due to growing conditions, species or variety, age, and analytical procedures used (Jones *et al*., 2020; Yan *et al*., 2020).

The canonical discriminant analysis (CDA) revealed three significant (p < 0.5) discriminant functions that accounted for 98.3 % of the variance in agave variety separation (Wilks' Lambda, F = 8.74, p < 0.0001, n = 36). The first two discriminant functions describe 95.3 % of the total variation. The FDC1 has the highest eigenvalue (72.85), describing 85.1 % of the variation caused by the ash content; the FDC2 has an eigenvalue of 8.76, expressing 10.2 % of the variation caused by the lignin content; and the FDC3 has 3.1 % of the variation caused by the cellulose content.

The interaction of discriminant functions 1 and 2 (Figure 2) results in the separation of agave varieties into five distinct groups. The PL and Sha varieties are classified as having an ash content of 7 to 7.6 %. Another group consists of the Chal and Man varieties, which vary in ash content from 4.9 to 5.9 %. Ayo, BC, and VC varieties are divided into separate groups. The Ayo variety contains the most ash (9.18 %), whereas BC contains the least ash (4.07 %) and lignin (13.54 %). Finally, the VC variety contains the highest levels of cellulose (71.35 %) and lignin (21.95 %).

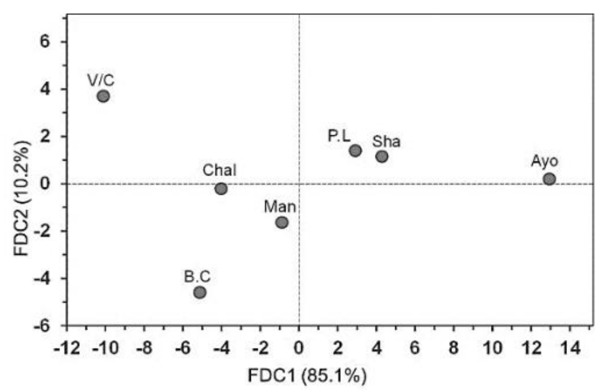

Figure 2. Centroids of two discriminant functions for the analyzed *Agave salmiana* species.

CONCLUSIONS

The BC, Man, and PL pulque maguey varieties share morphological characteristics, including wide leaves and long apical spines. The Ayo variety is the tallest. The canonical discriminant analysis revealed differences in the stalks' chemical composition, particularly in the levels of ash, lignin, and cellulose, the latter two of which are critical in the energy industry. The varieties in this study have a high cellulose percentage (61–71 %), and they can be used to produce bioethanol, particularly the BC variety, which has the lowest lignin percentage and one of the highest cellulose percentages. Although the VC variety is morphologically similar to Chal, the canonical discriminant analysis distinguishes it from the other varieties by containing the highest percentage of lignin and cellulose.

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CROP SENSITIVITY TO DICAMBA AND 2,4-D APPLIED AT COMMERCIAL AND SUBDOSE LEVELS

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ABSTRACT

The development of dicamba and 2,4-D resistant crops may result in the widespread use of these herbicides in agricultural areas, potentially affecting nearby susceptible crops. This study aimed to evaluate the sensitivity of bean, peanut, and cotton to different doses of Dicamba and 2,4-D. To do so, separate experiments were conducted for each crop and herbicide in a greenhouse, using a completely randomized design with four replications. Both herbicides were applied at different doses to plants with the second pair of true leaves. Phytotoxicity and dry biomass were evaluated. For cotton, 2,4-D showed high phytotoxicity at doses of up to 83.75 g ai ha^{-1}, with a reduction in suppressive effect observed at 20.93 g ai ha^{-1}. Dicamba exhibited a pronounced reduction in crop biomass up to a dose of 70 g ai ha^{-1}, with 94 % phytotoxicity. For beans, the evolution of symptoms using 2,4-D occurred more slowly, but the highest doses resulted in phytotoxicity of up to 95 % and a 30 % reduction in biomass at 167.5 g ai ha^{-1}, indicating a significant impact on the crop. Dicamba also had a high negative impact, with a 100 % reduction in biomass at 70 g ai ha^{-1}. The peanut crop was more tolerant to herbicides, with 2,4-D doses of 670, 335, and 167.5 g ai ha^{-1} resulting in phytotoxicities of 69.5, 37.49, and 14.37 %, respectively. Dicamba significantly reduced dry biomass at doses of up to 70 g ai ha^{-1}. The results show that, despite the differences in sensitivity of cotton, bean, and peanut to 2,4-D and Dicamba, even low doses of these herbicides applied early in development had a significant negative impact on these crops.

Keywords: *Phaseolus vulgaris, Arachis hypogaea, Gossypium hirsutum*, phytotoxicity, hormonal herbicides, contamination.

INTRODUCTION

Synthetic compounds acting as phytohormonal "superauxins" have been effective herbicides in agriculture for over 60 years. These so-called auxin herbicides are more stable in plants than the main natural auxin, indole-3-acetic acid (IAA), and have systemic mobility and selective action. They are primarily used to control dicotyledonous weeds in cereal crops. These herbicides belong to different chemical

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classes, such as phenoxycarboxylic acids, benzoic acids, pyridinecarboxylic acids, carboxymethyl aromatic derivatives, and quinolinecarboxylic acids (Grossmann, 2010).

Concerns about synthetic auxin herbicides stem from increased use of 2,4-D and Dicamba in several countries, including Brazil, as a result of recent transgenic crops releases. In 2019, at least 9 million kg of Dicamba were applied to soybeans and cotton in the United States, either before sowing or during the growing season, whereas less than 5 million kg were applied to all other cropping systems and pastures (USGS, 2019).

Currently, more than 90 % of the soybeans grown in Brazil are transgenic, with tolerance to glyphosate and insect attacks. The National Biosafety Technical Commission (CTNBio), an agency responsible for approving genetically modified organisms in Brazil, released a soybean cultivar resistant to Dicamba in 2016 to increase the number of technologies available to farmers. In addition to Dicamba-tolerant soybean, CTNBio has been marketing soybean resistant to 2,4-D, glyphosate, and ammonium glufosinate since 2019 (CTNBio, 2022). While the new technology helps manage weeds, especially for glyphosate-resistant species, herbicides can cause damage to sensitive crops due to drift or residue contamination in sprayer tanks (Behrens *et al*., 2007; Riter *et al*., 2021; Tian *et al*., 2023).

2,4-D is the first modern herbicide and has been used on farms, roads, and lawns since the late 1940s (Peterson *et al.*, 2016). The Brazilian Health Regulatory Agency (ANVISA), an agency linked to the Ministry of Health, has reassessed 2,4-D and will maintain it in the Brazilian market with restrictions on its application, such as preventing the same worker from preparing and applying the product when a tractor is used and setting crop-specific time intervals for worker entry into treated areas. These measures are consistent with recent reassessments of the herbicide in other countries. Currently, 2,4-D is available worldwide except in Mozambique, where its use is prohibited (ANVISA, 2019).

Dicamba has been used for weed control in corn, wheat, pasture, and turfgrass plantations since the 1960s, and its sale in Brazil began in the 2021-2022 growing season. Its use is recommended in the pre-planting of soybeans and cotton and in post-emergence applications for soybean crops with the Intacta2 Xtend™ technology, which metabolizes the herbicide into non-toxic compounds. Symptoms of auxin herbicides include inhibition of cell division, obstruction of phloem flow, epinasty (bending) of leaves, petioles, branches, and stems, changes in leaf venation and wrinkling, brittle stems, chlorosis, wilting, and necrosis of leaves, and alteration of growth and atrophy of roots. The death of susceptible plants occurs slowly, ranging from 3 to 5 weeks after application (Vidal and Merotto, 2001).

Some older formulations of 2,4-D and Dicamba were known to be highly susceptible to spray drift and post-application volatilization, which can lead to irreversible problems, as reported by Mueller *et al.* (2013), Everitt and Kelling (2009), and Marple *et al.* (2007). Even at very low doses, auxin herbicides may have collateral effects (da

Silva *et al.*, 2018), and several crops are highly sensitive, such as non-Dicamba-resistant soybean, beans, cucumber, beet, tomato, cotton, and peanut, as reported by Johnson *et al.* (2012) and de Aguiar *et al.* (2020).

To reduce the potential for external movement, legal proceedings and government regulations resulted in changes in the way these herbicides are used and in the physicochemical characteristics of their formulations (Miller and Butler-Ellis, 2000). However, 2,4-D and Dicamba were reported as first and third, respectively, on the list of herbicides that caused crop damage due to drift in the US. Farmers in 25 American states have filed more than 2700 complaints with state agricultural agencies regarding Dicamba damage to conventional soybean and other crops (AAPCO, 2017).

Under field conditions in Florida, dose-response studies have shown that peanut plants treated with 2,4-D (doses ranging from 70 to 1120 g ai ha^{-1}) and Dicamba (from 35 to 500 g ai ha^{-1}) had phytotoxicity from 0 to 35 and 20 to 78 %, respectively, as reported by Leon *et al.* (2014). Dicamba volatilization in the post-emergence application was reported as the primary exposure route, causing damage to non-target vegetation (Sciumbato *et al*., 2004). Cotton plants subjected to subdoses (0, 1/200, and 1/400 of the commercial dose (CD) of 561 g ai ha^{-1}) of 2,4-D and Dicamba at different phenological stages showed higher phytotoxicity at the 3–4 leaf stage at the time of application, and higher plant recovery was observed in Dicamba-treated plants, as reported by Marple *et al.* (2008).

Dicamba drift on 'Ponkan' mandarin seedlings was more toxic compared to 2,4-D, but both herbicides severely affected the physiological processes of the plants, according to Brochado *et al.* (2022). Lettuce was highly sensitive to 2,4-D drift, resulting in plant death with doses of up to 1/8 of the commercial dose, while Dicamba provided phytointoxication levels higher than 30 % for up to 1/8 CD. The tomato crop also showed high susceptibility up to subdoses of 1/32 for both herbicides (Roesler *et al.*, 2020).

Contamination with 2,4-D and Dicamba can arise when residues in sprayer tanks are not properly washed. When these residues come into contact with other herbicides, solvents, or adjuvants, they can dilute and cause contamination (Roesler *et al.,* 2020). It is crucial to distinguish plant responses resulting from particle drift, volatilization, and tank contamination in the field because the management approaches for each are fundamentally different (Riter *et al.,* 2021).

Therefore, to address the imminent problems related to the large-scale use of auxin herbicides, this study aims to evaluate the effects of different doses of Dicamba and 2,4-D on commercial cotton, bean, and peanut crops that are sensitive to these herbicides. The hypothesis is that different doses of auxin herbicides can cause initial damage to crops that are not tolerant to them.

MATERIALS AND METHODS

The experiments were carried out in a greenhouse at the Federal University of São Carlos, Brazil, using bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*), and cotton

(*Gossypium hirsutum*) as the cultivated species. The experimental design involved a completely randomized setup with four replications, and each crop was evaluated separately for two herbicides (2,4-D and Dicamba) at nine different doses, including control treatments without herbicide application. The herbicide doses tested were 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.791, and 0 % of the commercial dose for each herbicide. Each experimental unit was comprised of a 5 L pot with a single plant. Crop varieties were selected based on their importance for producers, including Netuno for bean, BS2106 GL for cotton, and Runner IAC 886 for peanut. Soil samples used in the experiment were subjected to chemical analysis (Table 1).

Table 1. Chemical analysis of Latossolo Vermelho-Escuro (Oxisol) samples used in the experiment.

Latossolo Vermelho-Escuro (Oxisol)									
P resin	OM	pH	K	Ca	Mg	H+Al	SB	CEC	V
Mg dm^{-3}	g dm^{-3}	$CaCl_2$			mmol dm^{-3}				%
15	38	5.6	5.4	53	13	26	71.4	97.4	73

To define the doses for the experiments, we consulted existing literature on simulated drift for the herbicides studied (Johnson *et al.*, 2012; Kruger *et al.*, 2012). Nine doses were used for each herbicide: 670 (commercial dose, CD), 335, 167.5, 83.75, 41.87, 20.93, 10.46, 5.23, and 0 g ai ha^{-1} for 2,4-D, and 560 (CD), 280, 140, 70, 35, 17.5, 8.75, 4.37, and 0 g ai ha^{-1} for Dicamba. In all three crops, plants were treated at the four-pair true leaf stage, with three tests conducted simultaneously. Spraying was carried out using a CO_2-pressurized knapsack sprayer at a constant pressure of 245.16 kPa, using an application boom equipped with nozzles with 110.03 fan spray tips and a spray solution volume of 200 L ha^{-1}. Relative humidity, air temperature, and wind speed during application were 70 %, 25 °C, and 4 km h^{-1}, respectively. After treatment, the plants were maintained in a greenhouse and irrigated as required.

Phytotoxicity was assessed at 5, 10, and 15 days after application (DAA) using a percentage score scale, in which 0 corresponds to no injury and 100 to plant death (ALAM, 1974). Shoot dry biomass was evaluated at 15 DAA, removing plants close to the soil surface, placing samples in paper bags, and drying them in a forced-air circulation oven at 65 °C until constant dry mass weight (about 72 h).

Dry biomass data were transformed into a percentage (%) relative to the control (without herbicide application), according to the following formula:

$$X\ (\%) = 100 - \left[\left(\frac{m\ rep\ treat\ x\ 100}{m\ \bar{x}\ test}\right)\right]$$

where X is the percentage reduction of the treatment, *m* is the mass (g), *treat* is the treatment, $\bar{x}$ is the mean, and *test* is the control.

The collected data (phytotoxicity and biomass reduction) underwent analysis of variance using the F-test. When significant, a regression analysis was performed to select the explanatory model of the dose-response curve based on statistical significance ($p < 0.05$) and determination coefficients (R^2) using SigmaPlot software (Systat Software Inc.).

RESULTS AND DISCUSSION

The herbicide 2,4-D presented a fast increase in phytotoxicity levels (Figures 1A and 1C), with low doses showing high phytotoxicity just five days after application. The commercial dose and half of it (670 and 335 g ai ha^{-1}) caused 90 % phytotoxicity at 15 DAA, leading to plant death. The dose of 83.75 g ai ha^{-1}, corresponding to 12.5 % of the commercial dose, resulted in a high phytotoxicity of approximately 90 %. The application of 41.87 g ai ha^{-1}, representing 6.24 % of the commercial dose, caused a progressive increase in phytotoxicity, reaching about 50 % in the last evaluation and potentially causing irreversible damage. The lowest doses of 2,4-D (20.93, 10.46, and 5.23 g ai ha^{-1}) did not show high phytotoxicity, presenting less than 10 % and causing only mild chlorosis in plants.

Cotton plants were sprayed with herbicide at the stage of four pairs of true leaves, i.e., in a juvenile phase. According to de Oliveira (2015), phytotoxicity in cotton plants caused by 2,4-D is inversely proportional to plant height, leaf number, and stem diameter. Hence, less developed plants showed more intense symptoms, which supports our results. Early application of 2,4-D in cotton crop development leads to injuries and negatively affects some cotton fiber quality characteristics, resulting in reduced productivity (de Souza, 2021).

Dicamba showed 79 % phytotoxicity at 5 DAA from 70.0 g ai ha^{-1} (12.5 % CD), which progressed to 94 % at 15 DAA, causing plant death (Figures 1B and 1D). Even lower doses (17.5 g ai ha^{-1}) resulted in approximately 17.5 % phytotoxicity, with visible symptoms characteristic of auxin mimetics such as epinasty, leaf bending, and necrosis. The lowest evaluated doses of 8.75 and 4.37 g ai ha^{-1} showed no significant injuries to the plants compared to the controls.

Neto (2019) reported that cotton plants may show symptoms such as leaf burn, necrosis, epinasty, leaf bending, and reduced development after 24 hours of indirect exposure to Dicamba using volatilization chambers, indicating a high potential for yield losses due to herbicide drift. Moreover, cotton is also susceptible to other auxin-mimicking herbicides, such as aminocyclopyrachlor. In this sense, Flessner *et al.* (2012) found that crop response to aminocyclopyrachlor drift was comparable to that of 2,4-D and aminopyralid, as treated plants showed injuries and reductions in height and shoot dry biomass, demonstrating the high susceptibility of cotton to this mechanism of action.

Cotton plant dry biomass reduction (Figure 1) followed a phytotoxicity response pattern. The highest 2,4-D doses (670, 335, and 140 g ai ha^{-1}) caused a biomass reduction of over 80 % in cotton plants. Doses of 83.75 and 41.87 g ai ha^{-1}, considered low (12.5 and 6.25 % of the field dose, respectively), showed a significant reduction ranging from 23 to 50 %, respectively. Plant biomass was not significantly affected by doses below 20.93 g ai ha^{-1}, although symptoms such as epinasty and leaf wrinkling were still observed. The two highest doses of Dicamba had the greatest impact on biomass, with a reduction of over 80 % at the highest dose. Intermediate doses of 140 and 70 g ai ha^{-1} significantly reduced biomass (75.10 and 86.55 %) compared to the control. Doses lower than 35 g ai ha^{-1} did not differ from each other, ranging from 4 to 12 % reduction.

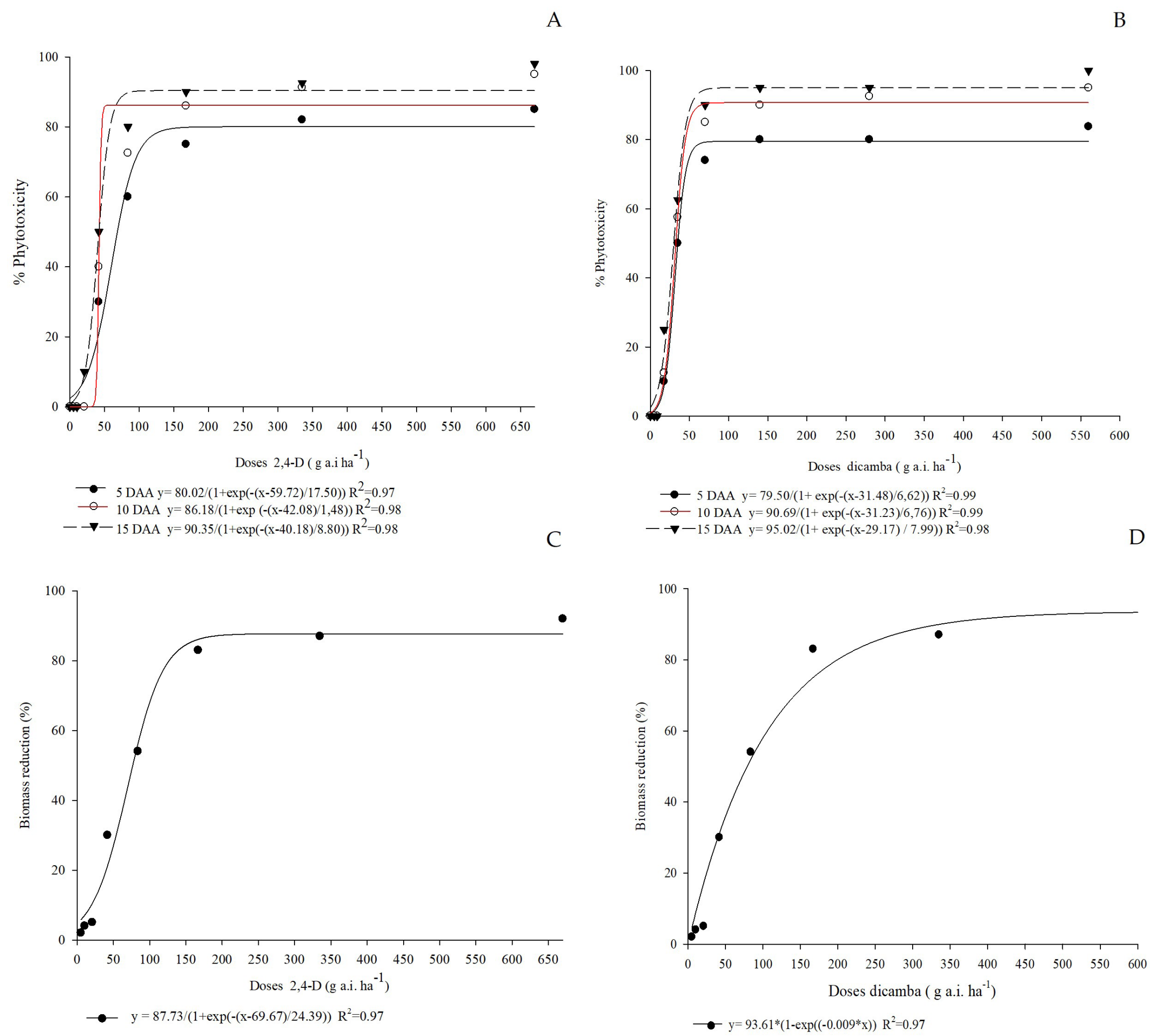

Figure 1. Phytotoxicity (%) and biomass reduction (%) on cotton plants at 5, 10, and 15 days after application (DAA). A, C: caused by 2,4-D; B, D: caused by Dicamba.

The phytotoxicity analysis of 2,4-D (Figure 2A) and Dicamba (Figure 2B), as well as biomass reduction for the bean crop (Figures 2C and 2D), show that the symptoms evolved more slowly for 2,4-D compared to the dynamics observed for cotton. The highest dose (670 g ai ha^{-1}) resulted in approximately 62 % phytotoxicity at 5 DAA, with visible symptoms of leaf yellowing and stem epinasty, progressing to 74 % at 10 DAA and 95 % at 15 DAA. Using half of the highest dose at 5, 10, and 15 DAA, the phytotoxicity reached 61.9, 72.9, and 84.2 %, respectively (Figure 2A).

Intermediate doses of 167.5 and 83.75 g ai ha^{-1} (25 and 12.5 % CD, respectively) resulted in final phytotoxicity of 55 and 35 %, respectively, indicating a high impact on the

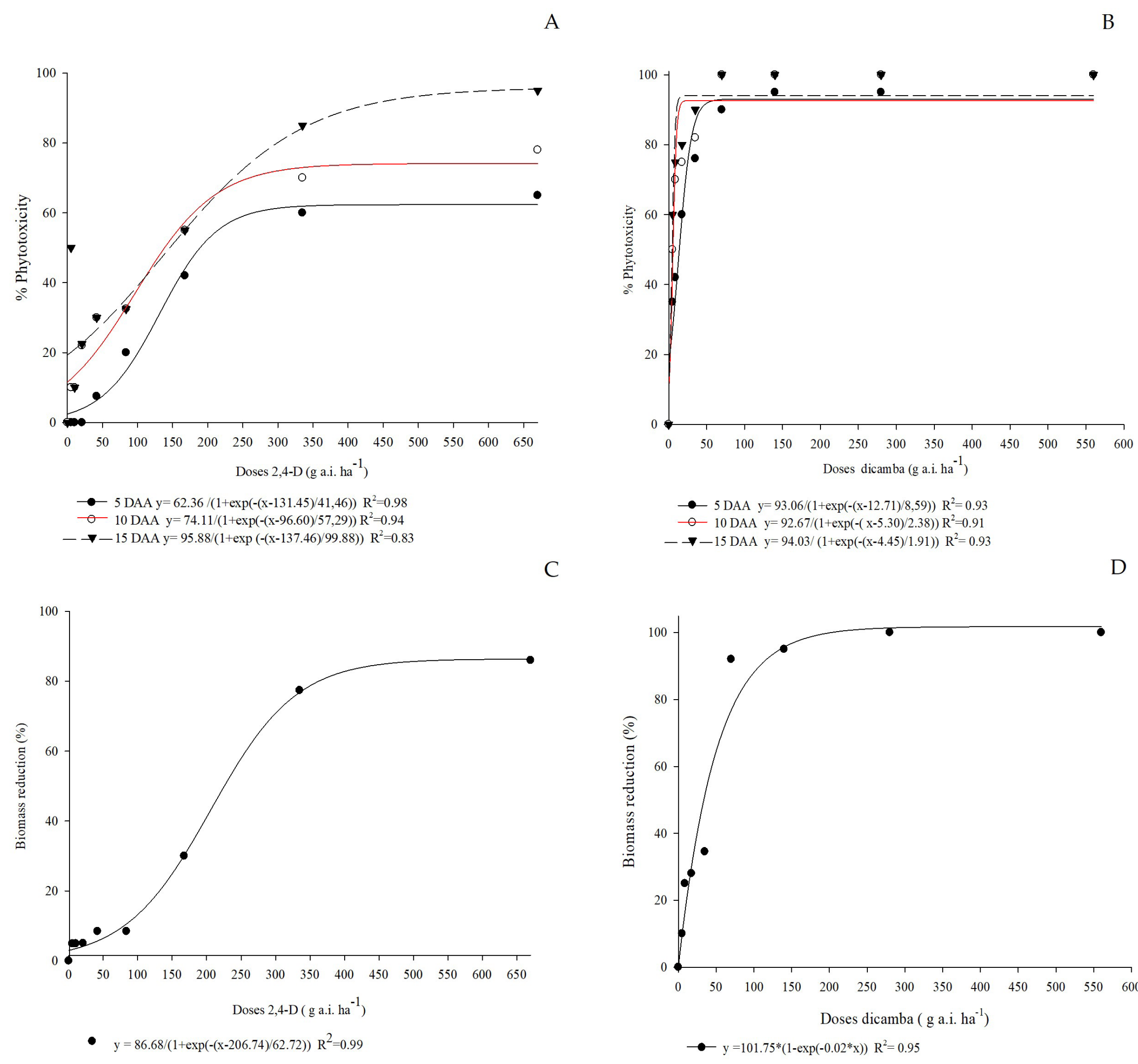

Figure 2. Phytotoxicity (%) and biomass reduction (%) on bean plants at 5, 10, and 15 days after application (DAA). A, C: caused by 2,4-D; B, D: caused by Dicamba.

crop. Although the phytotoxicity dropped as 2,4-D doses decreased, slight chlorosis was still observed in plants even with the minimum dose (5.23 g ai ha^{-1}), causing 20 % phytotoxicity. Massucato *et al.* (2021) found that bean seedlings subjected to subdoses of 2,4-D showed a reduction in germination and length, and increasing concentrations reduced emergence and increased plant injuries.

The evolution of phytotoxicity caused by Dicamba in the bean crop was observed in the first evaluation, with little or no difference between evaluation times for most doses. Phytotoxicity reached approximately 86 % in the initial evaluation (5 DAA) even at low doses (6.25 % CD). At 15 DAA, values were above 85 %, with the exception of the lowest dose of 4.37 g ai ha^{-1} (0.78 % CD), which had a phytotoxicity of 46 %. Thus, high injury rates were observed even at the lowest doses, with higher doses resulting in plant death and intermediate doses resulting in severe and irreversible damage to beans. In their study, de Aguiar *et al*. (2020) found that the dose to inhibit 50 % of bean plants was 17.75 g ai ha^{-1} at 14 DAA, indicating that the species, together with soybean, is extremely sensitive to Dicamba.

The impact of Dicamba on bean biomass (Figure 2D) clearly showed two groups of doses: 280 to 560 g ai ha^{-1} resulted in a total reduction in plant biomass, while 4.37 to 35.0 g ai ha^{-1} reduced plant biomass by 15 to 50 %. Thus, doses above 35 g ai ha^{-1} can be considered highly problematic for the crop. The two highest doses of 2,4-D reduced bean dry biomass by 85.99 and 77.37 %, respectively, making them the most harmful to the crop. The dose of 140 g ai ha^{-1} (25 % CD) caused a reduction of 23 %, while the other doses showed biomass reductions ranging from 0 to 10 %. Hence, the biomass reduction data shows that Dicamba has a higher negative impact on the crop when applied at this stage of development.

The results of phytotoxicity and biomass reduction caused by 2,4-D (Figures 3A and 3C) and Dicamba (Figures 3B and 3D) in the peanut crop show that phytotoxicity symptoms increased with the course of the evaluations, with epinasty in the stems, especially at doses above 83.75 g ai ha^{-1}, but with no high suppressive effect as in the other crops. The evaluation conducted at 5 DAA showed phytotoxicities of approximately 34.9, 10.3, and 2.57 % for doses of 670, 335, and 167.5 g ai ha^{-1}, respectively. These values evolved to 69.5, 37.49, and 14.37 % at 15 DAA, respectively. Doses lower than 20.93 g ai ha^{-1} resulted in mild chlorosis, which culminated in phytotoxicity values below 5 %. Similarly, Leon *et al.* (2014) observed that peanut plants sprayed with 2,4-D showed low phytotoxicity. On the other hand, Faircloth and Prostko (2010) reported that 2,4-D applied 75 days after peanut planting affected crop yield due to phytotoxicity.

Peanut plants sprayed with doses of 560, 280, 140, and 70 g ai ha^{-1} of the herbicide Dicamba showed phytotoxicity ranging from 44.9 to 48.2 % at 5 DAA, with symptoms of leaf and stem epinasty. The phytotoxicity reached values ranging from 72 to 83.6 % at these same doses at 15 DAA. Therefore, the variation between the percentages from 100 to 12.5 % CD was low. Doses of 35, 17.5, and 8.75 g ai ha^{-1} showed lower values, ranging from 10.8 to 35.4 %.

According to Blanchett *et al.* (2015), peanut plants become more sensitive as they approach the reproductive stage (initial flowering), making them more susceptible to herbicide damage at advanced stages of development. There is a direct relationship between the dose of a product and the sensitivity of the plants, reflecting the damage, which can lead to a reduction in productivity. In this study, the applications took place when the peanut plants were at the beginning of their development, thus showing greater tolerance to underdosing. Leon *et al.* (2014) also observed that Dicamba doses of 70, 140, 280, 560, and 1120 ai ha^{-1} resulted in phytotoxicity ranging from 20 to 78 % in the peanut crop, reducing its productivity by 65 %.

Results on the reductions in shoot dry biomass of peanut plants treated with 2,4-D and Dicamba (Figure 3) show that the highest dose of Dicamba led to a reduction of 59.15 % in plant dry biomass, while the dose of 70 g ai ha^{-1} showed a 24 % reduction,

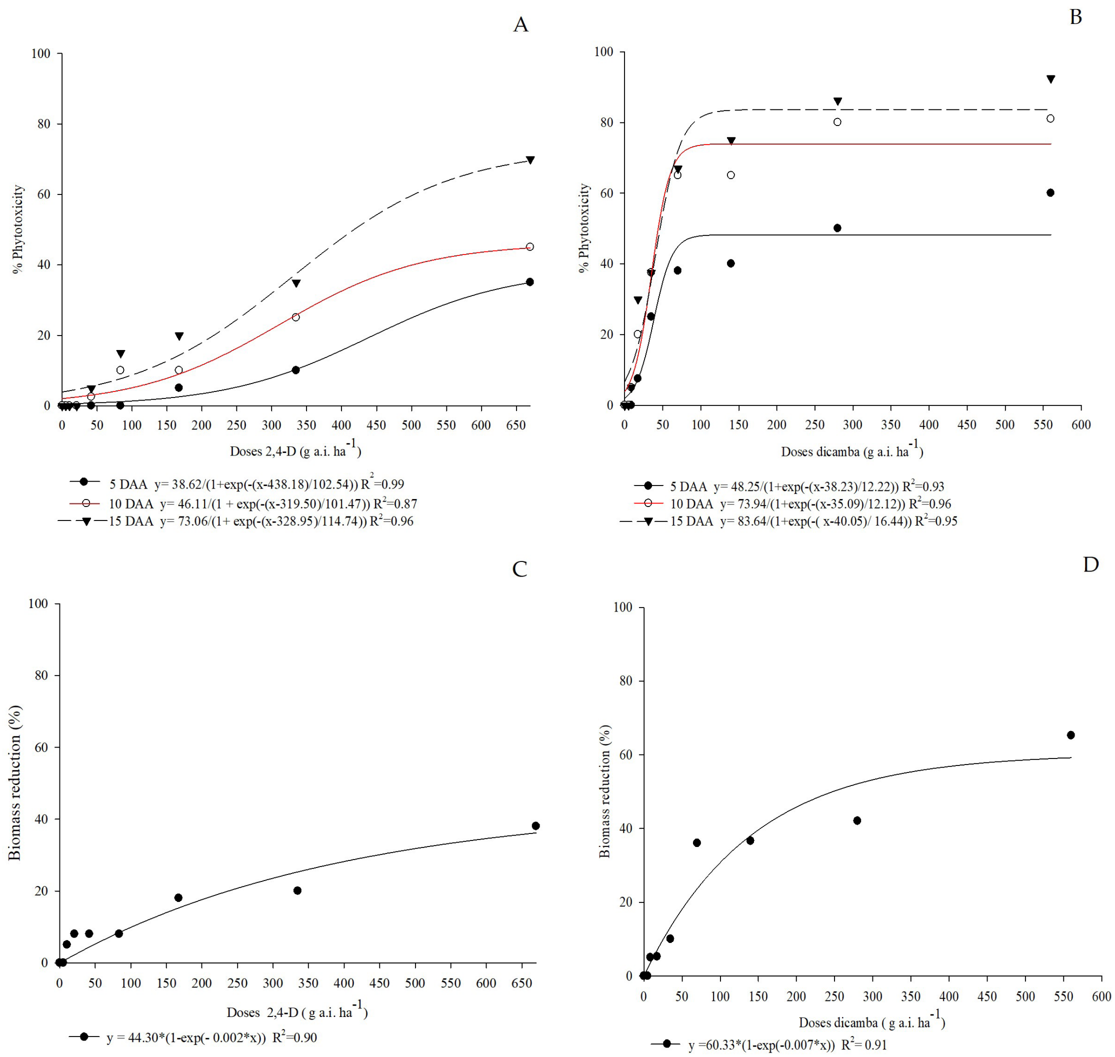

Figure 3. Phytotoxicity (%) and biomass reduction (%) on peanut plants at 5, 10, and 15 days after application (DAA). A, C: caused by 2,4-D; B, D: caused by Dicamba.

which is half of the commercial dose. The lowest doses had a smaller impact on plant biomass (11.2 % reduction) at the dose of 35 g ai ha^{-1} and no reduction at 4.37 g ai ha^{-1}. The highest reductions in dry biomass of peanut plants treated with 2,4-D were observed at doses of 670, 335, and 167.5 g ai ha^{-1}, with values ranging from 36.5 to 15 % reduction. The other doses reduced relative biomass by less than 10 %, showing a higher tolerance of plants.

It is important to highlight that hormonal herbicides require special care when applied, as they have a high potential for drift. Some municipalities even establish normative instructions for the use of these herbicides. A safety border area must be adopted between the application area and sensitive crops, in addition to observing the meteorological conditions at the time of application, the risks of thermal inversion, and other precautions to prevent potential risks of drift and volatility.

CONCLUSIONS

Low doses of the herbicides 2,4-D and Dicamba can affect cotton, beans, and peanuts at an early stage of development and therefore have a significant negative impact on these crops. Among the species evaluated, the peanut crop required higher doses of herbicides to cause phytotoxicity. These herbicides should be avoided near areas with these crops.

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Agrociencia

EFFECT OF DIFFERENT SPATIAL ARRANGEMENTS OF *Jatropha curcas* L. ON FOOD CROPS

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ABSTRACT

The objective of this study was to assess, during three years, the spatial arrangement that will enhance the morpho-productive development of *Jatropha curcas* L. in association systems with staple crops. It was hypothesized that the association of crops is a factor that determines the behavior of the species and the development of agroenergetic farms, considering rainy and low rainfall periods. A quasi-experimental design was used, and four treatments were tested: 1) *J. curcas* (Jc) in monoculture; 2) 50 % of the area with Jc and 50 % planted with food crops in rotation (CA); 3) Jc intercropped with CA; and 4) area planted with CA. A principal component statistical analysis was performed with the variables fruit yield and associated crop yield using 95 % confidence intervals from a Student's t-test. The number of bunches and total fruit and seeds harvested from Jc were shown to be the most important and had a positive correlation for the first component in each period. As for fruit yield, the highest values were achieved in monoculture. Although high yields were not attained in CA, the relationship is regarded favorable since the use of polycultures is viable, fundamentally in the arrangement of 50 % Jc + 50 % CA, being positive the biological efficiency given by the diversification and the best equivalent land use.

Keywords: monoculture, intercropping, agroecosystem, food security.

INTRODUCTION

Jatropha curcas L. belongs to the *Euphorbiaceae* family and is a perennial shrub native to Central America (Mitra *et al*., 2021). The seeds of this species have a high oil content. They are considered non-edible due to the presence of toxic compounds harmful to humans and animals (Zimila *et al*., 2021). This oil can be used to produce biofuel and other by-products. In addition, the residual cake after being detoxified can be used for feeding different animal species and the husk as a source of energy due to its caloric potential (Figueroa-Saavedra *et al*., 2020).

Several authors (Valdés-Rodríguez *et al*., 2013; Ávila-Soler *et al*., 2018) highlight that *J. curcas* supports land reclamation and develops in different soils and agroclimatic conditions, with seed yields varying between 0.5 and 5 Mg ha^{-1} per year. However, after a decade of studies on the species, this information is considered imprecise and heterogeneous. Abobatta (2019) emphasizes that *J. curcas* is still in the process of domestication, that its behavior is similar to that of other perennial crops, and that to reach peak production, it is necessary to take into account varietal selection, certain specific soil, climate, and agronomic management conditions.

Among the factors to be considered in agronomic management, plant density is one of the elements that most influence agricultural yield. All crops require an optimal area to capture adequate light energy that favors photosynthesis and increased leaf growth, which can lead to increased productivity (Alvarado-Ramírez *et al*., 2022). Different planting densities are used in plantations of *J. curcas*, depending on the objectives pursued. In monoculture of the species, up to 5000 plants ha^{-1} have been used (Góngora-Canul *et al*., 2018). Other low densities are also used, fundamentally in association systems with food crops, which is a practice based on the policies of environmental sustainability and food security, since diverse products are obtained in the same space and better use is made of the soil.

Müller *et al*. (2014) determined a greater development of crown diameter and number of *J. curcas* shoots when using 6 x 3 m spacings in two types of associations: agrosilvopastoral (maize-*Brachiaria-Jatropha* integration) and silvopastoral (*Brachiaria-Jatropha* integration). On the other hand, in studies conducted in Mexico by Rucoba-García and Munguía-Gil (2013), when comparing two production systems of *J. curcas* in monoculture and in association with *Zea mays* L. and *Phaseolus vulgaris* L., it was possible to determine the economic profitability of intercropping the tree with these crops. It was demonstrated that with intercropping, the profits were slightly higher than those obtained with monocultures.

In Cuba, research has been carried out on the association of more than twenty food crops, in which considerable production has been achieved, mainly of beans (*P. vulgaris* L.), soybeans (*Glycine max* (L.) Merr.), peanut (*Arachis hypogaea* L.), corn (*Z. mays* L.), cassava (*Manihot esculenta* Crantz), and sorghum (*Sorghum bicolor* (L.) Moench), with average productivity rates of 3.5 kg of fruit per tree of *J. curcas* (Sotolongo-Pérez *et al*., 2007), which has made it possible to assess the adaptation of the plant to the soil and climatic conditions of the country, to be considered in production systems from the inclusion in agroenergy farms. However, the results of these studies are diverse, and in many cases, the yields represent unreliable extrapolations.

The aim of this study was to assess the spatial arrangement that will enhance the morpho-productive development of *J. curcas* in association with food crops. Under the hypothesis that spatial distribution is one of the agronomic management factors that determine the morpho-productive behavior of *J. curcas* associated with food crops, its study will allow the evaluation of the most appropriate variants for the development of agroenergetic farms.

MATERIALS AND METHODS

Geographical location and experimental period

The experiment was conducted at the Experimental Station of Pastures and Forages "Indio Hatuey" (22° 48′ 7″ N and 81° 2′ W), at 19.01 m altitude, in the municipality of Perico, Matanzas province, Cuba. The experiment lasted for three years, from November 2016 to October 2019.

Edaphoclimatic characteristics

The experiments were established on a flat topography soil with a slope of 0.5 to 1 % and of the genetic type Ferrallitic Red Leached (Hernández-Jiménez *et al*., 2015). The average depth to bedrock is 150 cm. The climate is classified as tropical sub-humid (Inzunza, 2005) and has two seasons defined by rainfall: the low rainfall period (PPLL) from November to April, and the rainy period (PLL) from May to October (Academia de Ciencias de Cuba, 1989).

During the experimental period (2016–2019), the yearly average temperature was above 24.1 °C. The precipitation volumes in 2018 were above the historical average (Figure 1). Data were taken from the Agrometeorological Station "Indio Hatuey" 78329, attached to the Institute of Meteorology (INSMET, 2016) and located at 19.9 m altitude in the municipality of Perico, Matanzas province, Cuba (22°, 48′, 7′′ N and 81° 2′ W).

Experimental procedure for *J. curcas*

J. curcas was planted three years before the start of the study to ensure stability in plant production (Lama *et al*., 2018). The soil was prepared conventionally (two plowing rounds were interspersed with two harrowing rounds), with an interval between tillages of approximately 20 days. Subsequently, two seeds from Cabo Verde were deposited in each hole. One year before the experiment, a homogenization cut was made, pruning at 10 cm above the base of the soil to remove the apical meristem and favor axillary branching and a greater quantity of fruits in all plants (Valdés-Rodríguez *et al*., 2020). On the other hand, food crops were intercropped between the *Jatropha* furrows: beans (*Phaseolus vulgaris* L.), sweet potatoes (*Ipomoea batatas* (L.) Lam.), and squash (*Cucurbita moschata* Duch), which were planted at different times, taking into account the climatic requirements of each species (Figure 2), and were associated according to the spatial arrangements that were evaluated.

In all cases, for the planting of food crops, soil preparation was carried out prior to planting, and two plowing and two harrowing operations were performed. For this, the technical standard for each crop (MINAG, 2012) was taken into account, and the agronomic variables necessary to estimate yield were evaluated.

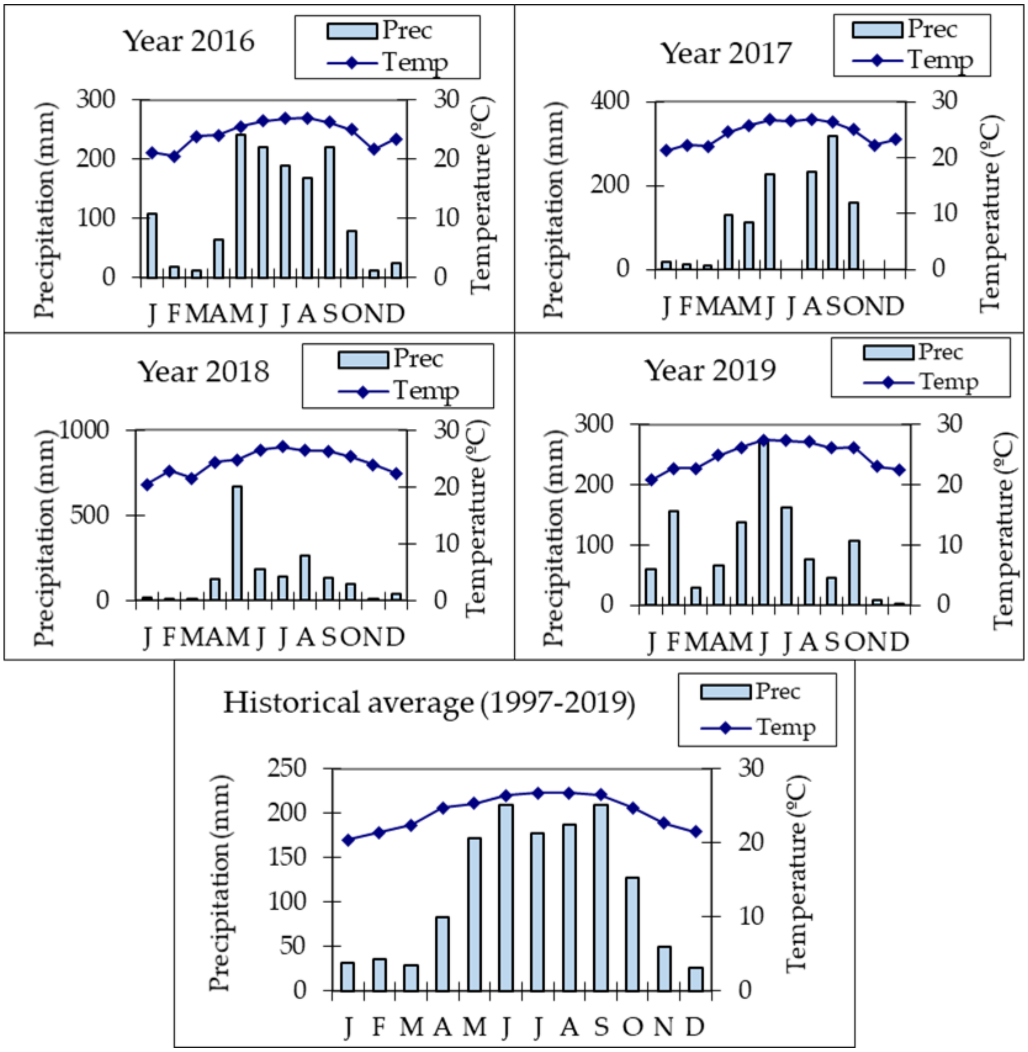

Figure 1. Behavior of climatic elements (mean temperature and precipitation) during the experimental period recorded at the Indio Hatuey Agrometeorological Station (INSMET, 2016).

Treatments and experimental design

Four treatments were studied (Table 1), and a quasi-experimental model was used as the treatments were applied in non-randomized plots (Curbeira-Hernández *et al.*, 2017). This design is employed when random assignment is impossible, and its purpose is to test the existence of a causal relationship between two or more variables (Bono-Cabré, 2012). For this, 15 *J. curcas* plants were considered replicates in each plot. The treatments were assigned as follows: T1, *J. curcas* (Jc) in monoculture, sown at 2.5 m between rows and 2 m between plants, composed of ten rows; T2, 50 % of the area with Jc sown at 2.5 m between rows and 2 m between plants, composed of five

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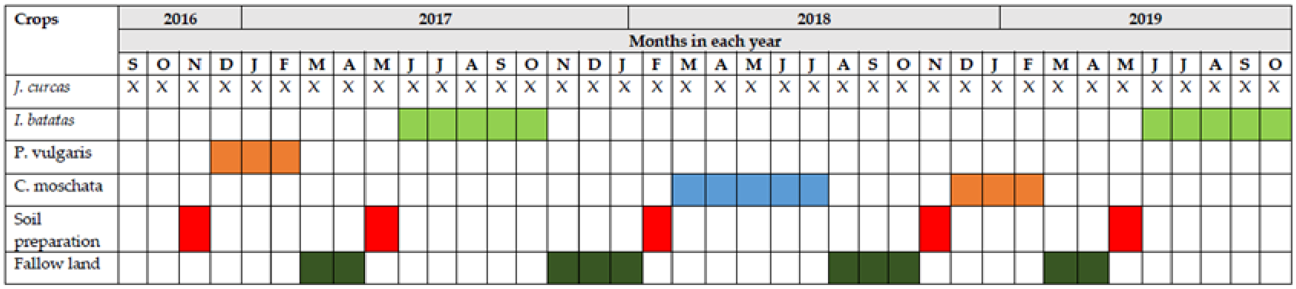

Figure 2. Period schedule of food crops associated with *Jatropha curcas* L. S: September; O: October; N: November; D: December; E: January; F: February; M: March; A: April; M: May; J: June; J: July; A: August.

Table 1. Jatropha curcas intercropping treatments with food crops.

Treatment	Spatial arrangement	Crop type	Evaluation year
T1	*J. curcas* in monoculture	-	2016–2019
T2	50 % *J. curcas* and 50 % with rotational cropping	Beans Sweet potato Squash	2016 and 2018 2017 and 2019 2018
T3	*J. curcas* intercropped with crops	Beans Sweet potato Squash	2016 and 2018 2017 and 2019 2018
T4	With food crops in rotation	Beans Sweet potato Squash	2016 and 2018 2017 and 2019 2018

rows and 50 % of the area sown with different food crops in rotation (Figure 2); T3, Jc sown at 5 m between rows and 2 m between plants, composed of five rows of the tree crop, intercropped with food crops in rotation; and T4, area sown with food crops in rotation. The factor under study was the spatial arrangement, which was evaluated to introduce *J. curcas* in different agroenergy systems (Figure 3).

Characteristics of association crop planting

In the case of beans, seeds of the Cuba-Cueto 25-9 black variety were used. Planting was done directly in the field, at 0.7 m between rows and 0.05 m between plants. Harvesting was done manually after 90 days (MINAG, 2012). For sweet potato, the INIVIT B 98-2 variety was used. Planting was carried out in the months of June 2017 and 2019 from cuttings of 25 to 30 cm in length, which were planted at 0.9 m between rows and 0.3 m between plants. Harvesting was carried out after 150 days, including all tubers (MINAG, 2012).

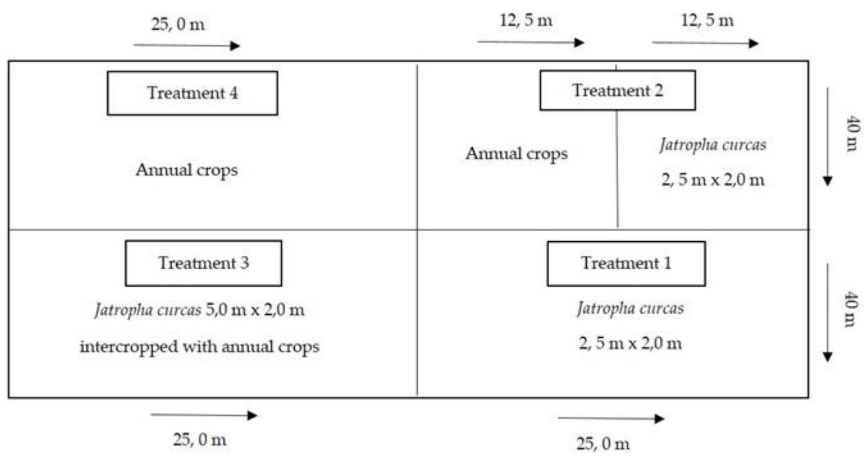

Figure 3. Distribution of the spatial arrangements (treatments) in the field.

Squash was planted in March 2019. Planting was done at 5 x 1 m between plants. The INIVIT C-88 variety was used. At sowing, two seeds were deposited per hole. When the plants had 2 to 3 leaves per plant, thinning was carried out, and the weakest plant was eliminated in each case. Harvesting took place after 120 to 150 days, when the fruits reached physiological maturity, that is, when the part in contact with the soil turned yellow (MINAG, 2012).

Evaluations in the cultivation *of J. curcas*

The evaluations were carried out each year, considering the two fruiting periods of *J. curcas* in the climatic conditions of Cuba, which are from July to September and from December to February, that coincide with the rainy and low rainfall periods. To carry out the morphological and productive measurements, the methodology proposed by Campuzano-Duque (2009) was used as a basis.

Morphological variables of *Jatropha*

The morphological variables were measured in each rainy period of each year when the plant began the reproductive phase: one month before each fruiting, in June (PLL), and November (PPLL). The variables recorded were: height (A), taken from the base of the plant to the apex of the main stem using a graduated ruler; stem thickness (GT) at a height of 10 cm from the soil surface, measured with a tape measure; crown diameter (DC), recording the horizontal length projected by the crown of the tree on the ground using a tape measure; and number of branches (NR), counting the number of branches inserted from the main stem.

Production variables of *Jatropha*

Harvesting was carried out in a staggered manner, as the fruits became yellow or black in color. Harvesting was carried out from July to September (PLL) and from December to February (PPLL) of each year. The following variables were counted: number of clusters (CR) formed on each plant; total fruit harvested (TFC), where the number of harvests in each period was determined by the level of fruit ripening; total seeds harvested (TSC), considering the number of seeds that each harvested fruit had per plant; and fruit yield (RF), considering the number of harvested fruits, their weight, and the number of plants per hectare.

Production variables of food crops

Yield was calculated considering five samples in each strip as replicates. The size of each sample was one linear meter and two furrows in beans and sweet potato, and for squash, it was a single furrow. In all cases, yield was calculated in Mg ha^{-1} from the sowing or planting frame used.

The biological efficiency of polyculture use was calculated to determine the feasibility of each spatial arrangement. For this purpose, the equivalent land use index (UET) was taken into account using the equation proposed by Casanova *et al*. (2001):

$$UET = Px / Ux + Py / Uy$$

where Px is the yield of crop x in polyculture, Ux is the yield of crop x in monoculture, Py is the yield of crop in polyculture, and Uy is the yield of crop in monoculture. If $UET > 1$, polyculture is advantageous; if $UET < 1$, it is not advantageous; and if $UET = 1$, the way of planting is indifferent.

Statistical analysis

For the data obtained, the relationship between morphological and productive variables was analyzed using multivariate statistics. For this purpose, the principal component analysis method was used, and the results were represented through a Biplot graph (Gabriel, 1971), where the treatments were plotted considering in each case the three years of evaluation and, within these, the PPLL and PLL periods. The fruit yield and associated crop yield variables were analyzed by descriptive statistics, using 95 % confidence intervals with the t-Student test statistic. The analyses were performed using the statistical package InfoStat 2008 (di Rienzo *et al*., 2011).

RESULTS AND DISCUSSION

The principal components of morphological and productive variables in *J. curcas*, as a result of different spatial arrangements in the first year of evaluation, are shown (Figure 3). During the PPLL, 100 % of the variance was explained (Figure 4A). The first component (CP1) extracted 61.5 % and height (A), stem thickness (GT), number of clusters (CR), total fruit (TFC), and harvested seeds (TSC) contributed to its formation, which were positively related, and the highest values were reached with the *Jatropha* monoculture (T1).

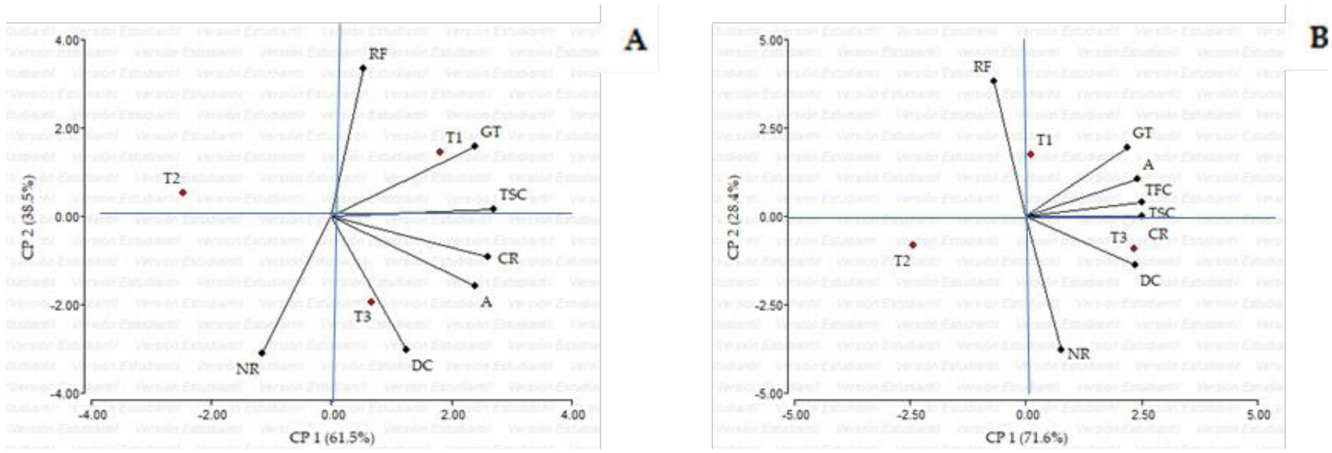

Figure 4. Principal component analysis (biplot graph) of morphological and productive variables by effect of spatial arrangements in *Jatropha curcas* L. in the first year of evaluation. A: low rainfall period (PPLL); B: rainy period (PLL). T1: Jc monoculture; T2: 50 % Jc + 50 % food crop; T3: Jc intercropped with food crop; A: height; DC: crown diameter; GT: stem thickness; NR: number of branches; CR: number of clusters; TFC: total fruits harvested; TSC: total seeds harvested; RF: fruit yield.

In the second component (CP2), a 38.5 % variance was detected, mainly explained by the fruit yield (RF) variables, and negatively related to the number of branches (NR) and crown diameter (DC); the highest values for the latter were reached when *J. curcas* was intercropped with food crops (T3), while for RF they were with T1.

For PLL, 100 % of the total variance was explained (Figure 4B). CP1 (71.6 %) was explained by A, DC, GT, CR, TFC, and TSC. For these variables, the highest values were obtained with T3. The variability extracted in CP2 was 28.4 % and is explained by RF and NR, which were negatively related. For RF, the highest values were found with the control treatment used for the tree (T1).

In the biplot representing the PPLL of the second year of evaluation (Figure 5A), a cumulative variance of 100 % was obtained. In CP1, a close positive relationship was detected between A, DC, NR, CR, TFC, and TSC. The highest values for these variables were found when using *J. curcas* intercropped with the associated crops (T3). In CP2 (29 % of the total variance), there was a negative correspondence between GT and RF, for which the highest values were reached with T1 (*J. curcas* in monoculture).

For PLL, 100 % of the total variance was also explained (Figure 5B). From CP1, 61.3 % was extracted. The variables that best explained this percentage were A, CR, TFC, and TSC, for which the highest values were reached when using *J. curcas* intercropped with food crops (T3). CP2 explained 38.7 % of the variance, and DC, NR, and, in the opposite direction to these variables, GT and RF interacted. For the latter, the highest values were found when using *J. curcas* in monoculture (T1).

In the low rainfall period of the third year of evaluation (Figure 6A), the total accumulated variance was again high at 100 %. For CP1 (76 %), the variables A, NR, CR, TFC, TSC, and GT contributed to fruit yield and were negatively related to the other

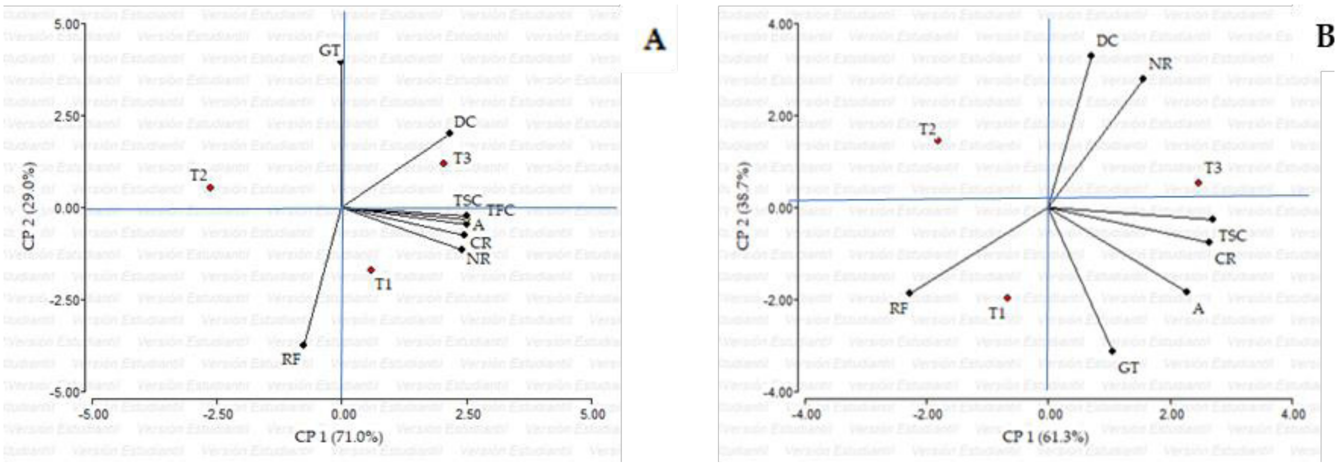

Figure 5. Principal component analysis (biplot graph) of morphological and productive variables by effect of spatial arrangements in *Jatropha curcas* L. in the second year of evaluation. A: low rainfall period (PPLL); B: rainy period (PLL). T1: Jc monoculture; T2: 50 % Jc + 50 % food crop; T3: Jc intercropped with food crop; A: height; DC: crown diameter; GT: stem thickness; NR: number of branches; CR: number of clusters; TFC: total fruits harvested; TSC: total seeds harvested; RF: fruit yield.

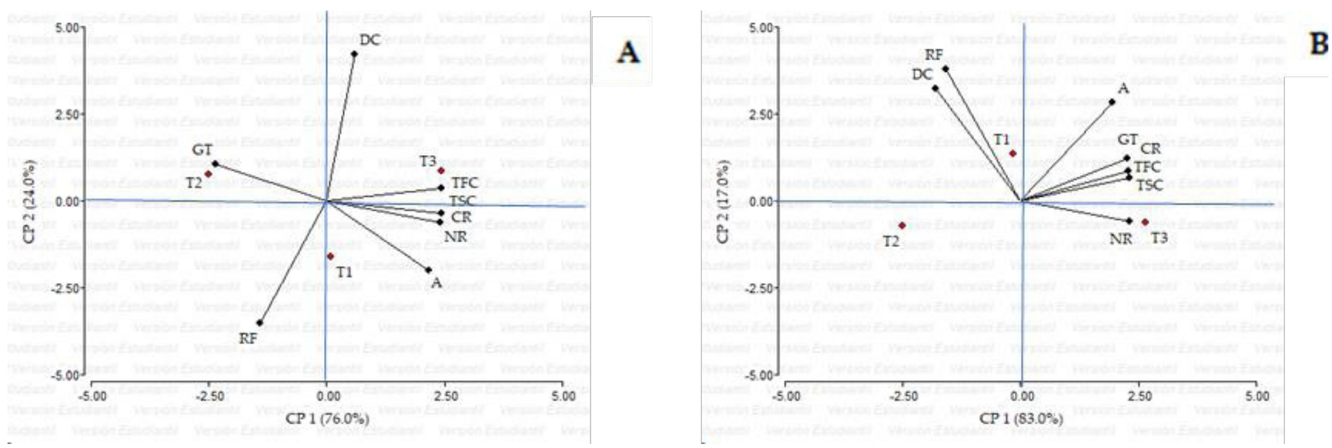

Figure 6. Principal component analysis (biplot graph) of morphological and productive variables by effect of spatial arrangements in *Jatropha curcas* L. in the third year of evaluation. A: low rainfall period (PPLL); B: rainy period (PLL). T1: Jc monoculture; T2: 50 % Jc + 50 % food crop; T3: Jc intercropped with food crop; A: height; DC: crown diameter; GT: stem thickness; NR: number of branches; CR: number of clusters; TFC: total fruits harvested; TSC: total seeds harvested; RF: fruit yield.

variables. The highest yields were achieved when using 50 % *J. curcas* and 50 % food crop (T2). However, for the former, they were higher with the spatial arrangement in which *J. curcas* was intercropped with food crops (T3). In CP2, 24 % of the variability was extracted; this component is explained by DC, and in the opposite direction, RF, for which the highest values were reached when using *J. curcas* in monoculture (T1). For the biplot corresponding to the PLL of the third year of evaluation (Figure 6B), the accumulated variance at that time was 100 %, which was explained by the first and second components, 83 and 17 %, respectively. In CP1, A, GT, NR, CR, TFC, and TSC contributed, which were positively related. Within this component, but with a negative relationship, there was DC. Furthermore, when using the spatial arrangement in which *J. curcas* was intercropped with food crops (T3), the highest values for A, GT, NR, CR, TFC, and TSC were reached. For CP2, RF was the variable with the greatest weight in the formation of this axis, for which the highest values were obtained with the tree control treatment (T1).

The results of the spatial arrangements effect on fruit yield (Figure 7) show that the values of this variable were higher when *J. curcas* was planted in monoculture (T1), with yields approaching or exceeding 2 Mg ha^{-1} at each harvest.

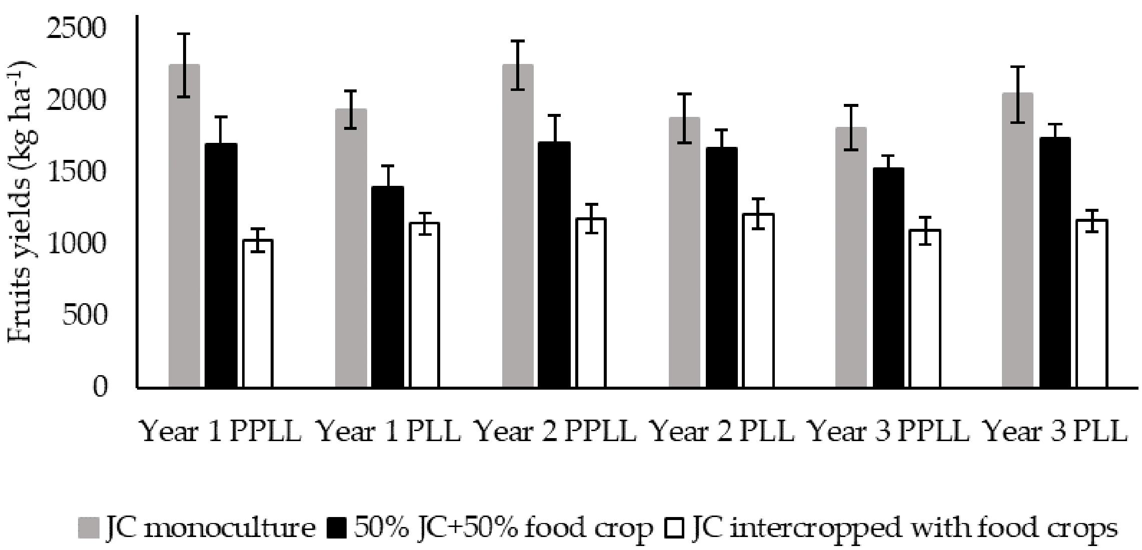

Figure 7. Effect of spatial arrangements on fruit yield of *Jatropha curcas* L. during two climatic periods in three crop years. Vertical lines indicate confidence intervals for each treatment. Results are not shown for T4 because it presents only food crops in rotation. PLL: rainy period; PPLL: low rainfall period.

The yield of the association of 50 % *J. curcas* and 50 % food crop (T2) was higher than that obtained with *Jatropha* intercropped (T3) and similar to T1 in the rainy period of the second year. Similarly, with T3 the most stable yields were obtained, since no differences were observed between the years evaluated.

Regarding the yields of food crops associated with *J. curcas* (Jc) during the experimental period, the results of each of the spatial arrangements in combination with Jc (T2 and

T3) and food crops in monoculture (T4) are reported (Table 2). For beans sown in two years (2016 and 2018), no differences were found between the spatial arrangements of 50 % Jc and 50 % bean (T2) and Jc intercropped with food crop (T3) relative to monoculture (T4). For T2, yields were slightly lower than the national average, which is 0.89 Mg ha^{-1} (ONEI, 2021).

Table 2. Effect of different spatial arrangements on the yield of food crops associated with *Jatropha curcas* L. during the experimental period.

Treatments*	Yield (Mg ha^{-1})				
	Beans		Sweet potato		Squash
	2016	2018	2017	2019	2018
T2	0.86	0.78	6.85	7.25	6.59
T3	0.91	0.84	8.25	8.37	11.89
T4	0.94	0.93	10.17	9.15	13.83
IC (±)	0.14	0.08	0.85	1.04	1.13

T2: 50 % Jc + 50 % food crop; T3: Jc intercropped with food crop; T4: monoculture of food crop; iC (±): confidence interval (95 %). *The results of treatment 1 are not shown since *Jatropha* was grown in monoculture.

Regarding the sweet potato crop, in 2017, differences were found among all the evaluated treatments. With the control (T4), the highest yield (10.17 Mg ha^{-1}) was obtained, being higher than the national average for the country (9.3 Mg ha^{-1}) (ONEI, 2021). However, in 2019, there were no differences between monoculture (T4) and when using *J. curcas* intercropped with the food crop (T3). For squash, the highest yields were obtained with monoculture (T4) and with *J. curcas* intercropped with food crop (T3), which did not differ between them but did when compared to T2 (50 % Jc + 50 % food crop), which had lower yields and was below the national average, which is close to 10 Mg ha^{-1} (ONEI, 2021).

The biological efficiency of the polycultures was positive given that the equivalent land use (UET) was higher in monoculture with respect to all the associations (Table 3). However, the highest values were obtained with the T2 spatial arrangement for most crops, except for squash, which was 1.35 in T2 and 1.50 in T3.

The productive variables number of clusters (CR), total fruit (TFC), and seeds harvested (TSC) had a positive correlation within the first principal component in each period, which indicated the high degree of complementarity existing in these productive traits. In addition, with the largest furrow spacing in an intercropping arrangement (T3), there were high values for each of these variables. This indicates that these association systems were effective over time.

Table 3. Equivalent Land Use (UET) behavior according to spatial arrangement variants.

Treatments*	UET				
	Beans		Sweet potato		Squash
	2016	2019	2017	2019	2018
T2	1.66	1.68	1.39	1.63	1.35
T3	1.43	1.50	1.40	1.47	1.50

T2: 50 % Jc + 50 % food crop; T3: Jc intercropped with food crop. *Treatments 1 and 4 are not presented because they were used as controls in the absence of intercropping in these spatial arrangements.

The shorter planting distance used in the *J. curcas* monoculture (T1) and T2 (50 % Jc + 50 % food crop) had the lowest CR, which could be associated with the reduced space between *J. curcas* plants and, therefore, a lower incidence of radiation, which can decrease photosynthetic activity and affect the productive stage. Meanwhile, the opposite occurred in the more open arrangements (Agustí, 2013). On the other hand, the variability in fruit yield (RF) was explained by CP2, and the highest values were obtained when *J. curcas* was used in monoculture (T1), which could be due to the high density of plants per hectare in this treatment. Therefore, it could be corroborated that the highest RF was followed by T2 (Figure 7), which responds to the same planting distance and plant density, compared to T1. The lowest height values (A) were obtained with treatments T1 and T2, so it was considered that a high plant density increases competition to assimilate necessary resources, which could be limited and influence growth from the first year of study (Rehling *et al*., 2021).

For crown diameter (DC), stem thickness (GT), and number of branches (NR), it was detected that these morphological characters were highly variable, as they were indistinctly represented in the two components in a positive or negative way, which could be determined by the genetic heterogeneity of the species (Rincón-Rabanales *et al*., 2016). The higher DC values with T3 during the first and second years of evaluation indicate that plants were able to reach their maximum vegetative development given a larger space between furrows (5 x 2 m), an aspect that varied once they reached full physiological maturity (Alegría-Muñoz, 2016).

The highest NR values were detected with T3. It is presumed that due to the more open spaces given by the greater separation between the arboreal furrows, the plants were able to develop a projected growth pattern in lateral branching. However, for GT, there was no clear trend for any treatment since, in each period, the behavior varied. In this sense, Agustí (2013) stated that the dilation or contraction of stem thickness is considered an important indicator of plant water status.

Considering these criteria, it was possible to infer that the tree was able to supply the water needs with the rainfall for each period, given the response expressed from the first year to the end of the evaluation period, since the thickness values did not fluctuate. These aspects corroborate that *J. curcas* reached maximum morphoproductive development when the rainfall range oscillated between 300 and 1800 mm (Kremer *et al.*, 2020), which corresponds to the accumulated rainfall for each year of the experimental stage. On the other hand, yields of beans, sweet potato and squash were considered low when compared to the national average for the country (ONEI, 2021). For squash, yields were less favorable due to the rainfall in 2018, which had accumulated 1688.6 mm, which was higher than the historical average of 1338.8 mm.

It is inferred that the lower yields for T2 may be associated with the spatial arrangement of the squash plants, which were unprotected and therefore the intense rains caused severe damage, while with T3, the associated plants were between the furrows of *J. curcas* that provided them with protection. Although high yields were not achieved in the food crops, it is considered that the association of crops is favored by the diversity of species and benefits provided by these plants. This evidence is corroborated by the positive equivalent land use (UET) value obtained for T2 and T3. Therefore, the use of associated crops was considered feasible, mainly in the arrangement of 50 % Jc + 50 % food crop (T2), which was superior to *J. curcas* intercropped with food crop (T3).

Not all species associated with *J. curcas* constitute viable systems, as it will depend on other factors characteristic of each food crop, such as growth habits, branching patterns, nutrient demands, and cultural requirements (Khanal *et al*., 2021). Therefore, it is important to determine the "ideal" spatial arrangements between different plant species in which responses can be obtained among the best associations, as well as to test with different organic fertilizers and investigate aspects of subsoil cover and planting distances (Valdés-Rodríguez *et al*., 2013; Valdés-Rodríguez *et al*., 2020), since the main objective is to achieve sustainability of agroecosystems and food security through agricultural biodiversity.

CONCLUSIONS

With the spatial arrangement of 50 % *Jatropha curcas* L. and 50 % food crops, the highest production of the tree was achieved. However, in the morphological variables, no clear trend in favor of any treatment or its relationship with yield was detected. The biological efficiencies (given by diversification and better land use) from the different associations of the *Jatropha* plant and associated crops were also positive.

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Agrociencia

GENETIC DIFFERENTIATION OF CULTIVATED CITRUS FRUITS (*Citrus* spp.) IN COLOMBIA USING SSR MOLECULAR MARKERS

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ABSTRACT

Citrus is the second most important fruit crop in Colombia after bananas, with production taking place in 26 of the country's 32 departments. Oranges, sour limes, and mandarins are the most economically important crops in terms of area planted and production. Microsatellites were used to assess the genetic differentiation of oranges, mandarins, tangelos, grapefruits, and acid limes from the Colombian Agricultural Research Corporation AGROSAVIA germplasm bank and collection of micro-grafted plants at Palmira Research Center in Colombia. A total of 121 samples from eight citrus groups were analyzed with 30 fluoromarked simple sequence repeats (SSR) microsatellites. The mean expected heterozygosity and mean observed heterozygosity were 0.58 and 0.57, respectively, and the coefficient of genetic differentiation was 0.558, confirming very high genetic differentiation among the citrus groups evaluated. Microsatellites mCrCIR01B02, AMB5, Ci01C09, mCrCIR08B08, and Ci01C07 were the most informative, presenting a high number of alleles and polymorphic loci percentages of more than 45 %; in addition, they allowed the identification of unique alleles, which can be used to establish the genetic fingerprint of citrus. Genetic differentiation was achieved for seven out of the eight groups evaluated. The SSRs used failed to differentiate the orange groups, possibly due to genetic origin; for this group, other molecular markers are recommended.

Keywords: Germplasm, genetic identity, characterization, planting material, fluorolabeled markers.

INTRODUCTION

Citriculture is one of the most important agricultural and economic activities in the world since these are the most productive fruit trees. Citrus fruits, due to their agronomic and commercial use, are grouped into four large varietal groups: 1) oranges; 2) mandarins; 3) grapefruit; and 4) limes and lemons. Sweet oranges lead world production with 56 %, followed by mandarins with 32 %, limes and lemons with 12 %, and grapefruits or pomelos with 7 % (FAO, 2020). Citrus is grown in more than 130 countries with tropical, subtropical, and borderline subtropical/temperate climate zones (FAO, 2020). In Colombia, citrus production in 2021 was 1 450 071 Mg with a harvested area of 87 638 ha and an average yield of 15 Mg ha^{-1} (MADR, 2021), demonstrating the economic importance of citrus genetic resources in the fruit industry, as they are produced commercially in 21 of the 32 departments in the country.

Citrus growers rely on both registered and unregistered nurseries with the Colombian Agricultural Institute (ICA) for the supply of planting material. Unfortunately, many of these nurseries do not have mother orchards to supply budwood and collect it from growers' farms, which poses a risk to the genetic traceability of the plants because of the possibility of mixing genotypes and the transmission of diseases through grafting. The citrus chain prioritized 15 cultivars, including oranges, mandarins, tangelos, grapefruits, and acid limes, as a bet of economic interest to develop the country's citrus industry.

In 2019, the ICA, through resolution 12816, established the requirements for the registration of nurseries and basic orchards, producers, and marketers of sexual and asexual seed (plant propagation material) of citrus. Nurseries are requested to ensure the genetic fidelity of the materials by using molecular markers as an alternative to the conventional method of morphological identification, which makes it difficult to distinguish between cultivars since some are differentiated by fruit traits and mother plants do not reach this phenological stage in nurseries.

In citrus, the use of molecular markers is useful for different purposes, such as the identification of hybrids and cultivars, characterization and classification of germplasm (García-Lor *et al*., 2015), relationships between canopies and rootstocks (Rohini *et al*., 2020), and obtaining genetic fingerprints, which allow detecting polymorphisms in DNA sequences and certifying the authenticity of the analyzed materials (Abdelaali *et al*., 2018; Chungada *et al*., 2021), since DNA profiles are not influenced by the environment.

One of the most widely used molecular markers are SSRs (simple repeated sequences). These markers are short DNA sequences consisting of motifs of 1 to 6 nucleotides repeated in tandem, of Mendelian inheritance. With a reliability higher than 95 %, they are co-dominant and highly informative. The number of repeats is variable and the degree of polymorphism increases with the total length of the microsatellite (Oliveira *et al*., 2002).

There are several methods used to determine the alleles generated by SSRs, one of which is the use of primers with fluorescent marking at the 5' end, which reduces genotyping errors and allows the use of several fluorochromes for the marking of the primers, allowing the detection by fluorescence emission of several markers in the same PCR product. This method consists of amplifying several SSR loci simultaneously, which requires the optimization of PCR conditions and selecting primers that, with similar hybridization temperatures, do not interact with each other, and with alleles in different size ranges (Abdelaali *et al*., 2018).

The objective of this study was to carry out genetic differentiation of oranges, mandarins, tangelos, grapefruits, and acid limes from AGROSAVIA germplasm bank and the collection of micro-grafted plants that are commercially grown, using fluorolabeled SSR markers. This research aims to contribute to the identification of citrus that will give rise to the mother plants of commercial varieties for nurseries registered with the competent authority within the citrus certification program in Colombia. It will also provide valuable information on the distribution of genetic variability and relationships within and among various citrus species.

It was hypothesized that citrus cultivated in Colombia is highly diverse and polymorphic in the 30 SSR loci analyzed. No studies have been conducted in Colombia to evaluate the genetic differentiation of citrus grown in different regions of the country using codominant molecular markers such as SSRs.

MATERIALS AND METHODS

Plant material collection

A total of 121 plants from 17 citrus accessions were selected from the germplasm bank and the collection of micro-grafted plants preserved in a protected environment in an aphid-proof net house located at the Colombian Agricultural Research Corporation AGROSAVIA, Palmira Research Center, Colombia. Each accession was represented by seven replicates; in addition, Eureka lemon and Rangpur lime accessions were included as comparison genotypes (Table 1).

Table 1. Citrus accessions used for the genetic differentiation study.

Number	Scientific name	Common name	Origin*
1	*Citrus reticulata* Blanco × *C. paradisi* Macf.	Minneola tangelo	Meta (Colombia)
2	*C. reticulata* Blanco × *C. paradisi* Macf.	Orlando tangelo	Corsica (France)
3	*C. clementina*	Clemenules mandarin	IVIA 22-19 (Spain)
4	*C. reticulata* Blanco	ICA Bolo mandarin	Palmira (Colombia)
5	*C. reticulata* Blanco	Oneco mandarin	California (USA)
6	*C. reticulata* Blanco	Arrayana mandarin	Quindío (Colombia)
7	*C. sinensis* (L.) Osbeck.	Campbell Valencia orange	California (USA)
8	*C. sinensis* (L.) Osbeck.	Frost Valencia orange	California (USA)
9	*C. sinensis* (L.) Osbeck.	García Valencia orange	Valle del Cauca (Colombia)
10	*C. sinensis* (L.) Osbeck.	Olinda Valencia orange	California (USA)
11	*C. sinensis* (L.) Osbeck.	Salustiana orange	California (USA)
12	*C. sinensis* (L.) Osbeck.	Sweety orange	Quindío (Colombia)
13	*C. sinensis* (L.) Osbeck.	Frost Washington orange	Valle del Cauca (Colombia)
14	*C. sinensis* (L.) Osbeck.	Valle Washington orange	Valle del Cauca (Colombia)
15	*C. aurantifolia* (Christm.) Swingle	Pajarito/Castillo acid lime	Quindío (Colombia)
16	*C.* × *latifolia* Tanaka ex Q. Jiménez	Tahití acid lime	Corsica (France)
17	*C. paradisi* Macf.	Star Ruby Grapefruit	California (USA)
18	*C. limon* (L.) Burm. F.	Eureka lemon	California (USA)
19	*C. limonia* Osbeck	Rangpur lime	California (USA)

*Source: SNBGVAA (National System of Plant Germplasm Banks for Food and Agriculture), Palmira Research Center.

DNA extraction

Citrus DNA was extracted from young leaves using the Doyle (1991) protocol. A 100 mg sample of tissue macerated with liquid nitrogen was resuspended with extraction buffer (100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0, 3 % CTAB (cetyltrimethylammonium bromide), 0.02 % B-Mercaptoethanol, and 1 % polyvinylpyrrolidone). The supernatant was washed with chloroform and precipitated with absolute ethanol and 5 M ammonium acetate. The pellet was eluted in deionized water treated with DEPC (diethylpyrocarbonate) and RNase (10 mg mL^{-1}). DNA was evaluated in terms of concentration (ng μL^{-1}) and quality (absorption spectra 260–280 and 260–230) by spectrophotometry using a NanoDrop™ 1000 (ThermoFisher, USA).

PCR conditions

Thirty SSR microsatellite markers (Table 2) were selected from studies conducted in different citrus populations (Oliveira *et al*., 2002; Novelli *et al*., 2006; Froelicher *et al*., 2008). The markers were fluorescently labeled at the 5′ end of the sense primer (Alpha DNA) and grouped into panels or multiplexes according to fluorochrome and amplification size (Table 2). The dye kit for SSR microsatellite labeling was DS-33 (Applied Biosystems, USA). The fluorochrome was selected based on the formation of panels where the amplification size and intensity of the bands at the time of visualization of the PCR products in 3 % agarose gels prior to labeling were taken into account. The markers with lower intensity were labeled with FAM and the rest with NED, VIC, and PET.

Table 2. SSR microsatellite markers used for citrus genetic differentiation analysis.

Primer name	Primer sequence F 5′-3′	Primer sequence R 5′-3′	Size (pb)	Melting temperature	Fluorophore
Ci01C07	TTG CTA GCT GCT TTA ACT TT	GTC ACT CAC TCT CGC TCT TG	236–278	55	FAM
mCrCIR01E02	TGA ATG GTA CGG GAA ATG C	CAG GGT CGG TGG AGA GGA T	153–175	53	VIC
mCrCIR01F04a	AAG CAT TTA GGG AGG GTC ACT	TGC TGC TGC TGT TGT TGT TCT	184–224	55	NED
mCrCIR08B08	GTG AAA GAG AGC AAG AAA AAT	AGC CAA AAA GAG AAG AAA TG	116–137	50	PET
Ci02A04	AAA GAA GTT AAA GAA AAA ATG	AGC GGT ATC GTA ATT CTC	156–165	55	FAM
mCrCIR01B02	AGC CAA AAA GAG AAG AAA TG	TTA GCA ATA TCA ACA TCA T	194–210	55	PET
Ci07C09	TAA GAT AAA AAC AGC ACA	GTG GCT GTT GAG GGG TTG	241–263	55	VIC
Ci07B09	TGC TGC TGC TGT TGT TGT TCT	AAA GAA GTT AAA GAA AAA ATG	186–196	50	NED

Table 2. Continue...

Primer name	Primer sequence F 5′-3′	Primer sequence R 5′-3′	Size (pb)	Melting temperature	Fluorophore
Ci01D11	TCG CTT TCT TAT TTC ACA CTC ACC	AGG ACA GAT GAC CCA GAT GAC A	197–210	55	FAM
Ci01G11	AAC ACC AAG AAG GAA GAG	TCG CTT TCT TAT TTC ACA CTC ACC	102–111	55	VIC
mCrCIR07H06	TTA TTT ATC TGT TTT CGC CTA	AGA TAC TTC ATT TGA TTG GC	180–194	56	PET
Ci01C09	GAC AGA ATG GGA GAG GAG A	TTG TCC CTT CCC TTT GTA	263–294	50	VIC
Ci02B07	CAG CTC AAC ATG AAA GG	TTG GAG AAC AGG ATG G	151–174	50	NED
Ci07C07	TAT CCA GTT TGT AAA TGA G	TGA TAT TTG ATT AGT TTG G	220–240	50	PET
mCrCIR06B07	CGG AAC AAC TAA AAC AAT	TGG GCT TGT AGA CAG TTA	91–109	50	FAM
Ci01D12	ATT TAT TTT CTT TCC CTT GTC TTA	TTT TCT TCT TCA TCT CTT TAC TGC	153–165	46	FAM
Ci02B10	TTT CAC AGC CAT CAC A	AAC ACC AAG AAG GAA GAG	180–200	50	PET
Ci07E06	AAT AAA CGC CCA CCT GAG AC	CAG TTG TTA AAA GGG AAG AAT GAA	223–244	55	NED
Ci08A10	GAGACTTTA CTTGAATGAA	ATCTCGTGTTGA AAATAA	152–165	50	VIC
Ci01H05	AAA ACA ACC AAA AGG ACA AGA TT	TTC AAA CTA AAC AAA CCA ACT CG	97–107	55	NED
Ci02F07	GCA GCG TTT GTT TTC T	TGC TGG TTT TCA GAT ACT T	169–200	55	PET
Ci06A05b	TCT CTG GTT GGT TTT TGT GA	ATG ATG AAA AGC AAG GGG	172–228	50	FAM
mCrCIR06A03	GGG TTG CGA CGA TGA GC	TCT CGG TTT GGC AGT TCG	231–237	55	VIC
Ci02F03	TAT CGA CAA CTC TTT CTC AT	TAA AGC GCA TGG ATA CT	158–171	55	PET
CCSM13	CTA GAG CCG AAT TCA CC	AAC AGC TAC CAA GAC ACC	160–177	53	NED
AMB5	CCC TGC ACA AAA ACT CAC AC	TGG GGG TGT TGA ATG GTA AT	108–143	56	FAM
AMB10	TAC TGT GGG GAA GGG ATC TG	GAC TCC TTC CAG CAC TTT GC	160–177	59	VIC
AMB8	TGA ACA TAT TTG CCC TTG GA	TTG TTT TGT GTG CTT GTG AGG	151–163	55	PET
CCSM6	ATC TGT GTG AGG ACT GAA	CCT CTA TTA ATG TGC CTG	204–248	51	FAM
CCSM147	AGA CTC ACG TAA CCT ACT TC	GCT ATG TTA TGA TAC GTC TG	109–133	54	VIC

pb: base pairs; F: forward; R: reverse.

For a final volume of 20 μL, 60 ng of DNA, 1.2 mM Buffer $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.8 mM dNTP's, 0.48 mM for each primer (sense and antisense), 0.2 X bovine serum albumin (BSA), 10 % trehalose, and 1 U Taq polymerase (recombinant) (Thermo Scientific, USA) were added. The amplification process was performed on an SureCycler 8800 thermal cycler (Agilent, USA) with the following thermal profile: 94 °C for 5 min, one cycle (94 °C for 45 s, 48–61 °C (2 °C above aligning temperature) for 45 s, 72 °C for 1 min), 35 cycles (94 °C for 30 s, 48–59 °C for 30 s, 72 °C for 1 min), 72 °C for 10 min, and 20 °C for 5 min.

After PCR, the amplified products were visualized on 1.6 % agarose gels, which were run in 0.5 X TBE buffer at 90 V and 400 mA for 60 min, stained with GelRed TM at a concentration of 13.3 X added to the sample, and visualized using an Enduro GDS gel photodocumenter (Labnet International, USA). The detection of fluorescent molecular markers was performed using an ABI 3730 xl automated sequencer (Thermo Scientific, USA) with a conformational analysis polymer.

Data analysis

The sequenced alleles were read using the bioinformatics program GeneMapper (Applied Biosystems, USA), which identifies the amplified microsatellite fragments as fluorescent peaks. The size of the alleles was estimated in base pairs, and homozygotes or heterozygotes were identified, as appropriate. The genetic parameters of number of alleles (NA), observed heterozygosity (Ho), expected heterozygosity (He), and private alleles per population were estimated with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006). The polymorphic information content (PIC) of each SSR microsatellite was calculated with PowerMarker version 3.25 (Liu and Muse, 2005). The coefficients of genetic differentiation (Fst), inbreeding coefficient within populations (Fis), and total inbreeding coefficient (Fit) were determined with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006).

To determine the kinship relationships between the populations evaluated, the Nei genetic distance (Nei, 1973) was estimated. The clustering analysis was performed using the UPGMA method with program TFPGA version 1.3 software. Genetic distances were visualized in three dimensions with principal coordinate analysis (PCoA) and calculated with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006). Genetic variability within and between populations was determined by analysis of molecular variance (AMOVA) using GenAlex version 6.5.0.1 (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

Population genetic diversity

A total of 7260 allelic data were obtained from the evaluation of 121 individuals, representing 19 citrus populations with 30 SSR markers, of which eight primer pairs were excluded for having more than 30 % null data (mCrCIR01F04a, mCrCIR07H06, Ci01D12, Ci08A10, mCrCIR06A03, CCSM13, AMB10, and AMB8). Analyses were performed with the remaining 22 markers, where 118 alleles (NA) were identified, with an average of five alleles per locus (Table 3). These results contrast with those obtained by Abdelaali *et al*. (2018), who evaluated seven SSR markers in a population of 22 citrus accessions, identifying a total of 44 alleles and six alleles per locus.

The mean observed heterozygosity (Ho) and mean expected heterozygosity (He) ranged from 0.07 to 0.93 and from 0.20 to 0.79, with means of 0.57 and 0.58, respectively. This indicates a high genetic diversity among the populations under study. The markers mCrCIR01B02, Ci01C09, AMB5, mCrCIR01E02, Ci02B07, Ci02F07, Ci06A05b, CCSM6, and Ci01C07 were the most informative, with Ho and He values higher than 0.67, which also presented the highest number of alleles. These results agree with those obtained by García-Lor *et al*. (2013), who studied the genetic organization of

Table 3. Genetic diversity parameters of 22 SSR microsatellite markers used in 122 cultivated citrus individuals.

Number	Microsatellite	NA	He	Ho	PIC	Fis	Fit	Fst
1	Ci01C07	7.00	0.79	0.91	0.76	-0.92	-0.05	0.45
2	mCrCIR01E02	6.00	0.73	0.79	0.69	-0.96	0.31	0.65
3	mCrCIR08B08	6.00	0.48	0.36	0.45	-0.97	0.03	0.51
4	Ci02A04	3.00	0.33	0.02	0.30	-0.67	0.54	0.72
5	Ci07B09	3.00	0.60	0.62	0.52	-0.93	0.52	0.75
6	Ci07C09	5.00	0.62	0.79	0.54	-0.97	0.10	0.54
7	mCrCIR01B02	7.00	0.68	0.73	0.63	-0.88	0.35	0.66
8	Ci01D11	4.00	0.46	0.05	0.39	-1.00	0.85	0.93
9	Ci01G11	2.00	0.20	0.19	0.18	-1.00	0.34	0.67
10	Ci01C09	6.00	0.71	0.89	0.67	-0.92	-0.14	0.41
11	Ci02B07	5.00	0.73	0.88	0.69	-1.00	-0.05	0.47
12	Ci07C07	3.00	0.46	0.19	0.41	-0.50	0.56	0.71
13	mCrCIR06B07	5.00	0.62	0.33	0.58	-0.84	0.44	0.70
14	Ci02B10	6.00	0.60	0.66	0.55	-0.96	0.09	0.53
15	Ci07E06	4.00	0.53	0.66	0.47	-1.00	-0.08	0.46
16	Ci01H05	5.00	0.26	0.07	0.24	-1.00	0.44	0.72
17	Ci02F07	5.00	0.74	0.71	0.70	-0.88	0.21	0.58
18	Ci06A05b	8.00	0.75	0.77	0.71	-0.97	0.26	0.63
19	Ci02F03	5.00	0.46	0.13	0.43	-0.94	0.55	0.77
20	AMB5	10.00	0.72	0.88	0.69	-0.98	-0.01	0.49
21	CCSM147	5.00	0.62	0.94	0.55	-0.93	-0.40	0.28
22	CCSM6	8.00	0.75	0.93	0.71	-0.98	-0.04	0.47
Average		5.36	0.58	0.57	0.54	-0.92	0.22	0.60
D.E		1.85	0.16	0.32	0.16	0.03	0.06	0.03

NA: number of alleles; He: expected mean heterozygosity; Ho: observed mean heterozygosity; PIC: percentage of polymorphic loci; Fis: inbreeding coefficient; Fit: total inbreeding coefficient; Fst: coefficient of genetic differentiation; D.E: standard deviation ($p \leq 0.05$), 10 000 permutations.

C. reticulata with 50 SSR markers, which included genotypes of *C. maxima*, *C. medica*, subgenus *Papeda*, *Fortunella* spp., *Microcitrus* spp., *Eremocitrus,* and *Poncirus trifoliata*, and obtained an average He of 0.71 with a range between 0.39 and 0.86, where Ci01C07, Ci02F07, Ci02B07, and mCrCIR01E02 presented He values higher than 0.57.

Polymorphic information content (PIC) ranged from 0.18 for marker Ci01G11 to 0.76 for marker Ci01C07 (Table 3). The average value for all markers was 0.54, indicating a medium level of discrimination. Similar values were reported by Chungada *et al*. (2021), who obtained an average PIC of 0.59 when evaluating in Nagpur (India) the genetic diversity of six citrus species with 14 SSRs. These results also coincide with those reported by Froelicher *et al*. (2008), who evaluated *C. reticulata* germplasm and obtained PIC values above 0.52. Conversely, Kumari *et al*. (2022) reported a PIC of 0.41 in 70 *C. aurantifolia* genotypes from the Indian breeding program.

In general, the Fst value for each of the SSR markers was high, with intervals between 0.276 and 0.927. The average value was 0.595 (DS = 0.032), which indicates very high genetic differentiation among the evaluated populations, according to Wright (1978). The average Fis value for the 22 markers was -0.918 (Table 3). The values ranged from -0.502 for marker Ci07C07 to -0.978 for marker Ci07C07. Negative Fis values were obtained for all 22 markers, indicating an excess of heterozygotes and that factors favoring variability are present in the populations studied.

In five of the 22 loci evaluated, we detected a total absence of homozygotes (Table 3). The total fixation index (Fit) obtained was 0.220 ± 0.064, with no significant differences between markers (p = 0.041); the values ranged from -0.006 to 0.854. These results contrast with those reported by García-Lor *et al*. (2013), who obtained an average Fit of 0.41.

Genetic distance

The dendrogram for the 19 populations constructed using the UPGMA method with a bootstrap of 10 000 replicates showed a high percentage of loci (95.45 %) supporting the formation of the nodes, which indicates a high reliability of the SSR markers used in the genetic differentiation analysis. Seven groups were formed in the dendogram. Group I included the orange populations (*C. sinensis*), Olinda Valencia, Valle Washington, García Valencia, Campbell Valencia, Salustiana, Frost Valencia, Sweety Orange, and Frost Washington, which were not genetically differentiated (Figure 1).

Citrus sinensis shows a high diversity in morphological, physiological, and agronomic aspects, which may be due to spontaneous somatic mutations (Barkley *et al*., 2006), mainly affecting fruit characteristics; however, very little genetic variation has been detected using molecular markers such as random amplified polymorphic fragments (RAPD) (Malik *et al*., 2012; Tripolitsiotis *et al*., 2013), inter simple sequence repeats (ISSR) (Tripolitsiotis *et al*., 2013), DNA amplification fingerprinting (DAF) (Luro *et al*., 2012), even with SSR microsatellite markers (Novelli *et al*., 2006; Ruiz *et al*., 2018).

This low genetic differentiation in the orange group is possibly due to the fact that they share ancestral hybrids that maintain low variability due to reproduction by apomixis

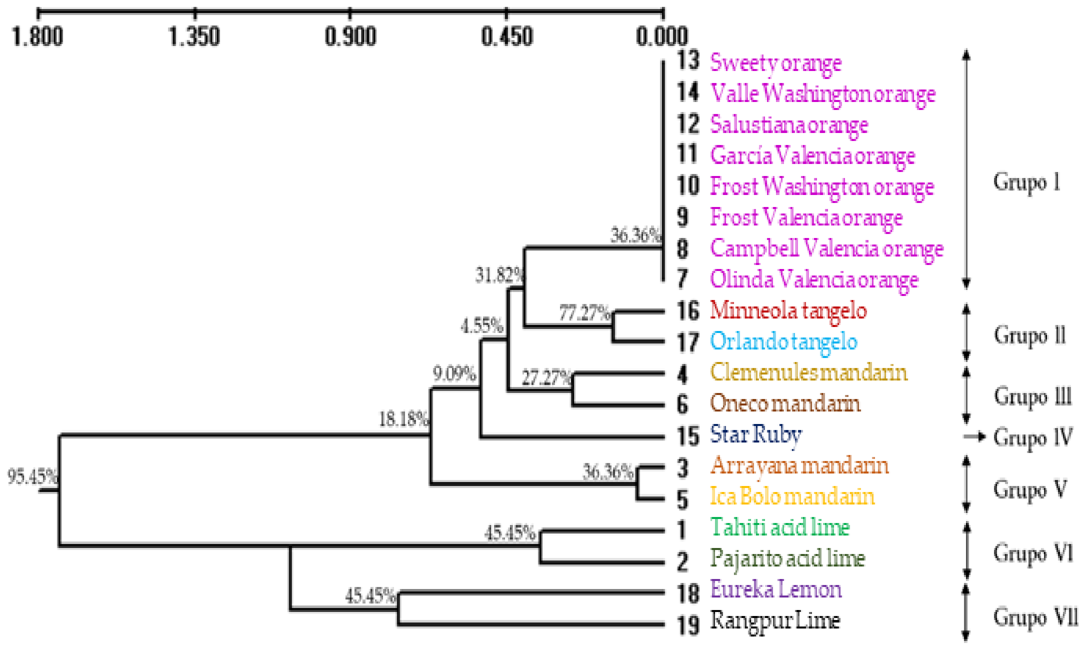

Figure 1. Nei genetic distances between the evaluated citrus populations, exemplified by a dendrogram constructed by using the UPGMA method, with a bootstrap of 10 000 permutations and the TFPGA program.

and that commercial variety multiplication is vegetative through the use of grafting (Shahnazari *et al*., 2022). Some authors have reported the use of molecular markers for genetic differentiation in *C sinenesis*. Juibary *et al*. (2021) reported the use of SCoT markers for the analysis of the structure and genetic fingerprint of 25 sweet orange cultivars from Iran, managing to identify some unique loci that can differentiate these cultivars. Similarly, Sagawa *et al.* (2018) reported the use of DArT seq markers as some efficient markers to identify genetic diversity at the species level in *Citrus*.

Group II consisted of Orlando and Minneola tangelos (*C. reticulata* Blanco × *C. paradisi* Macf.). These populations had a disimilarity index of 0.144. The mandarins Oneco and Clemenules were located in group III with a dissimilarity of 0.261, and they were clearly differentiated from Ica Bolo and Arrayana, which were associated in group V. *Citrus reticulata* exhibited high genetic variability, as determined by morphological characters and molecular markers (Froelicher *et al*., 2008; Tripolitsiotis *et al*., 2013; Suárez-Contreras *et al*., 2020). Star Ruby grapefruit (*C. paradisi* Macf.) was placed in group IV.

When comparing the interspecific genetic distances of the genotypes evaluated, Star Ruby was found to be genetically distant from Tahiti acid lime (*C.* × *latifolia* Tanaka ex Q. Jimenez) (1.943) and Pajarito acid lime (*C. aurantifolia* (Christm.) Swingle) (1.894), but showed a closer genetic relationship with the orange group with a dissimilarity index of 0.430. The SSR analysis revealed a genetic affinity among the orange, mandarin, tangelos, and grapefruit group (Figure 1), possibly explained by their phylogenetic and common ancestral relationships. *C. reticulata* has been considered as one of the main ancestral groups of cultivated citruses, along with *C. medica*, *C. maxima*, and *C. micrantha* (Nicolosi *et al*., 2000; Wu *et al*., 2018). *C. reticulata* includes gene introgression into other species, such as tangors (*C. reticulata* × *C. sinensis*) and tangelo; moreover, mandarin has been proposed to be a parent of *C. sinensis*. Roose *et al*. (2009) suggested that *C. sinensis* has 75 % *C. reticulata* and 25 % *C. maxima*, indicating backcross 1 (BC1) (*C. maxima* × *C. reticulata*) × *C. reticulata*.

This same hypothesis was also postulated in the orange genome sequencing work developed by Xu *et al*. (2013). However, this theory differs from the hypothesis of Nicolosi *et al*. (2000), who proposed that orange originated from a direct hybridization between *C. maxima* and *C. reticulata*. García-Lor (2013) contradicts these hypotheses and propose that the two orange parents are interspecific hybrids. Their results show that *C. sinensis* has three-allele genes that appear to be solely inherited from the *C. maxima* gene cluster and eight-allele genes from *C. reticulata*. Meanwhile, Star Ruby grapefruit has shared alleles from the *C. maxima*, *C. reticulata,* and *C. sinensis* gene pools (Luro *et al*., 2012).

Group VI included acid limes (Tahiti and Pajarito), which showed a dissimilarity of 0.351 and were genetically distant from the rest of the genotypes; this could be partly explained by the fact that they do not share common ancestors. According to molecular data, *C. aurantifolia* is a hybrid between *C. medica* and *C. micrantha* (Nicolosi *et al*., 2000; Ollitrault *et al*., 2012), while *C. latifolia* is a triploid hybrid between *C. aurantifolia* and *C. limon* (Rouiss *et al*., 2017), hence the close genetic relationship between these two species.

Group VII included Eureka lime (hybrid between *C. aurantium* × *C. medica*) and Rangpur lime (hybrid between *C. reticulata* × *C. medica*) with a dissimilarity index of 0.759, which showed closer genetic relationships with acid limes and a high genetic differentiation with the rest of the citrus populations. These results agree with those obtained by Nicolosi *et al*. (2000) and Kumar *et al*. (2022), who found close phylogenetic relationships between *C. medica*, *C. aurantifolia,* and *C. limon.*

The PCoA principal coordinate analysis in the first three coordinates explained 62.44 % of the total variability of the 19 citrus populations (Figure 2). The first coordinate explained 38.03 % of the genetic variability of the sour lime populations (Tahiti and Pajarito) and the oranges of the rest of the populations. The second coordinate explained 24.42 % and separated the Oneco, Ica Bolo, Arrayana, and Clemenules mandarin populations. Tangelos (Orlando and Minneola) were more closely related to the mandarin group, and grapefruit was located close to oranges, which can be explained by their phylogenetic relationships and common ancestors.

In general, citrus genotypes exhibited a distinctive genetic background; the greatest dispersion was observed among the acid limes in comparison to the rest of the populations because they do not share a common origin. The orange cultivars showed

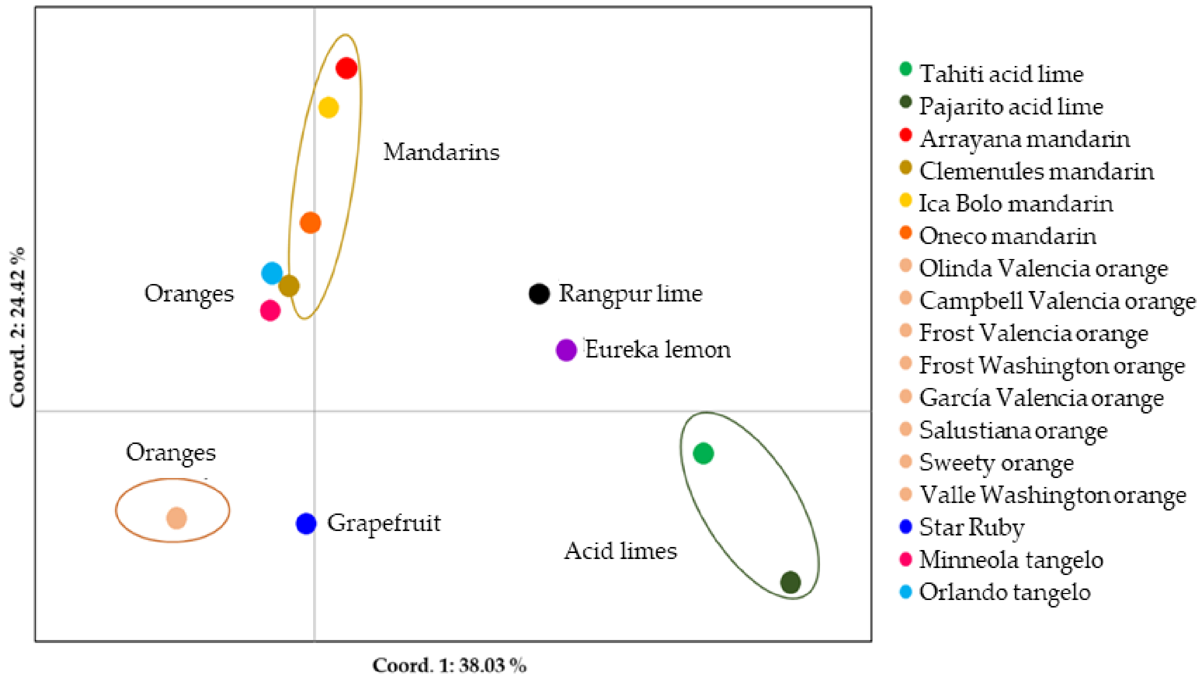

Figure 2. Two-dimensional principal coordinate analysis (PCoA) of genetic variation (Nei genetic distance) among citrus genotypes.

a high intragroup affinity, suggesting that they have a very close genetic basis. In contrast, the mandarin group was more variable. The reference species Eureka lemon and Rangpur lime showed closer relationships with the acid limes.

Genetic differentiation (Fst)

According to the Wright (1978) scale, acid limes presented Fst values above 0.442 when compared with oranges, mandarins, tangelos, and grapefruit, which indicates a very high genetic differentiation between these populations. The mandarin cultivars Arrayana and Ica Bolo showed intermediate genetic differentiation (Fst = 0.133), while Oneco and Clemenules had very high genetic differentiation (Fst = 0.253) (Figure 3). The Orlando and Minneola tangelos also showed high genetic differentiation (Fst = 0.185); however, the orange cultivars of the seven groups evaluated could not be genetically differentiated with the use of these microsatellites, so some other molecular markers could be used, such as single nucleotide polymorphisms (SNP) and insertion or deletion markers (InDel), which have been used by other authors for the genetic differentiation of orange germplasm (Ferrer *et al*., 2021). For other citrus groups, these results confirm the discriminatory power of SSR microsatellites and agree with García-Lor *et al*. (2013), who were able to demonstrate a high degree of genetic differentiation between the different citrus taxa evaluated.

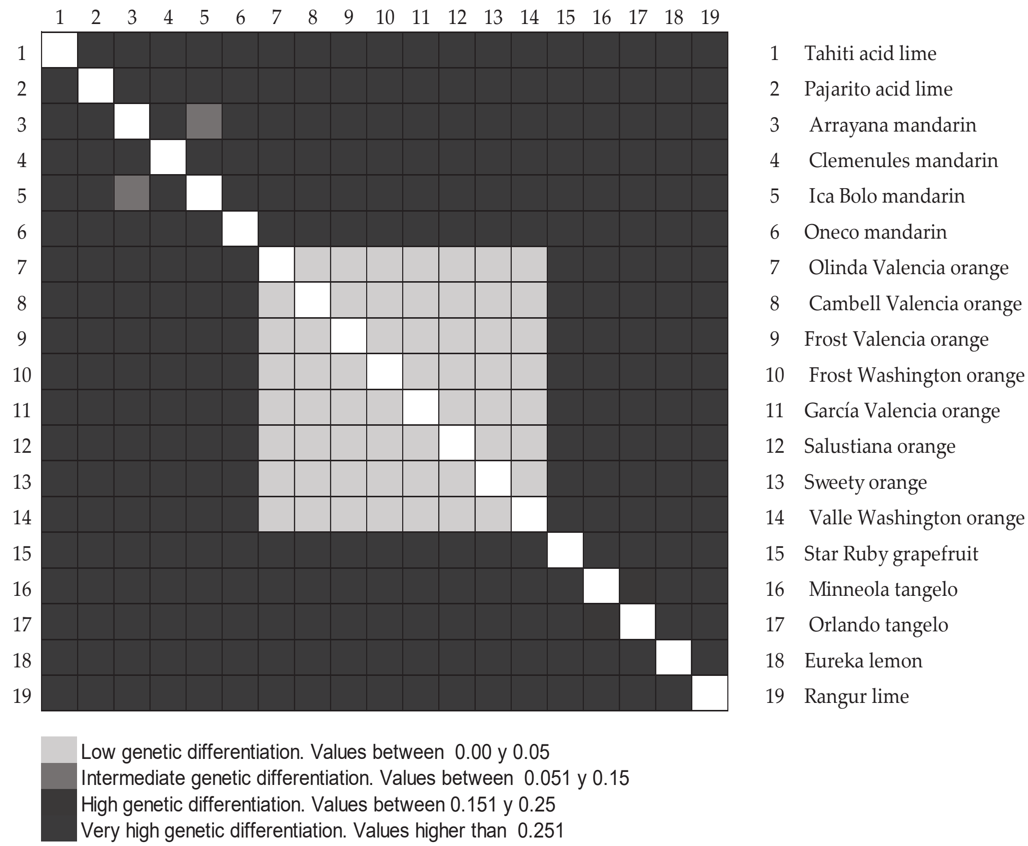

Figure 3. Heat map from Fst values among the evaluated populations. The darker the color tone, the greater the genetic differentiation between populations. **Figure 3**. Heat map from Fst values among the evaluated populations. The darker the color tone, the greater the genetic differentiation between populations.

Analysis of molecular variance

The results of the AMOVA revealed that the greatest percentage of variation corresponded to differences between populations (89 %), followed by the variation between individuals within populations, which corresponded to 11 %, with significant (p = 0.041) and non-significant values (Table 4). The variation within populations did not show significant differences (p = 1.0), due to the asexual reproduction method used to obtain the plants. The 17 citrus varieties micrografted were obtained from the germplasm bank plant collection using shoot-tip grafting *in vitro*, and thus are considered clones.

Single alleles

The number of single alleles was estimated for Tahiti acid lime, Pajarito acid lime, Arrayana mandarin, Clemenules mandarin, Oneco mandarin, Star Ruby, Eureka lemon, and Rangpur lime cultivars (Table 5).

Table 4. Analysis of molecular variance (AMOVA) for the 19 citrus populations.

FV	G.L	SM	CM	S^2 (%)	Fixation indexes
Between populations	18	543.26	46.586	89	Fst: 0.595 **
Between individuals within the population	104	84.208	0.745	11	Fis: -0.918 ns
Total	122	627.47	6.870		

FV: Sources of variation; G.L.: Degrees of freedom; SM: Sum of squares; S^2: Total variance; CM: Mean square; *P-value based on 10 000 permutations, ***p* < 0.0001; ns: not significant.

Table 5. Single alleles obtained in citrus cultivars by SSR markers.

Cultivar	Markers	Single alleles
Tahití acid lime	mCrCIR01B02, Ci02B10, AMB5	3
Pajarito acid lime	mCrCIR01B02, Ci07E06, Ci02B10, AMB5, CCSM6	5
Arrayana mandarin	AMB5	1
Clemenules mandarin	Ci01C09	1
Oneco mandarin	mCrCIR08B08	1
Ica Bolo mandarin	AMB5	1
Star Ruby grapefruit	mCrCIR08B08, Ci02A04, Ci07C09, mCrCIR01B02, Ci02B10, Ci01C09	6
Minneola tangelo	Ci01C07, Ci01C09	1
Orlando tangelo	Ci01C07	1
Eureka lemon	Ci01C07, Ci01H05, AMB5	3
Rangpur lime	Ci07C09, mCrCIR01B02, Ci01D11, Ci01C09, Ci01H05, Ci02F03, AMB5, CCSM6	7

The mCrCIR01B02, AMB5, Ci01C09, mCrCIR08B08, and Ci01C07 markers identified single alleles in sour lime, mandarin, grapefruit, Eureka lemon, and Rangpur lime. These SSR microsatellites were very informative, presenting a high number of alleles and a polymorphic information content above 45 % (Table 5), which can be suggested as genetic fingerprinting patterns (Chungada *et al*., 2021).

CONCLUSIONS

There is a high genetic diversity among citrus (*Citrus* spp.) cultivated in Colombia. The fluoromarked SSR microsatellite markers used in this study allowed genetic differentiation of the groups of mandarins, tangelos, grapefruits, and acid limes within and between species. Oranges exhibit little genetic differentiation. These markers can be used in studies of genetic diversity and variability, as well as to establish barcodes as genetic fingerprints of citrus in germplasm banks and populations with special agricultural characteristics, which can be used for genetic improvement programs or the implementation of strategies for the conservation of genetic resources.

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GENERATING NITROGEN RECOMMENDATIONS FOR MAIZE: ECONOMIC CONSIDERATIONS

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ABSTRACT

The use of excessive amounts of nitrogen fertilizers in crops usually results in nitrogen (N) losses, increasing environmental pollution and contributing to climate change, while also elevating crop production costs. This study aimed to determine in maize (*Zea mays* L.): (1) the economic optimal nitrogen rate (EONR), economic optimal yield (EOY), and N use efficiency (NUE) utilizing the current N/maize (grain) price ratio (N/MPR) for attainable yield levels; and (2) the effect of an increase in the N/MPR on the EONR, EOY, net income (NI), NUE, and the loss of N. Data from 140 maize response experiments to increasing rates of N, conducted between 2010 and 2019 across 18 states in Mexico under rainfed and irrigated conditions, were analyzed. In these experiments, the average maximum yields were 4.971 ± 2.716 Mg ha^{-1} under rainfed conditions and 11.776 ± 2.366 Mg ha^{-1} under irrigated conditions. The EONR, EOY, and NUE were determined with the current N/MPR for attainable yield levels. At these levels, both the EONR and NUE increased with higher EOY and decreased with increased yield without N treatment. An increase in N/MPR reduced EONR and its associated costs without significantly affecting EOY and NI. The reduction in EONR resulted in higher NUE and, consequently, lower N losses. By utilizing N/MRP in economic optimization, recommended N rates for maize can be reduced, leading to decreased N losses and environmental pollution.

Keywords: *Zea mays* L., economic optimal nitrogen rate, nitrogen/maize price ratio, economic optimal yield, nitrogen use efficiency.

INTRODUCTION

Given the extensive global cultivation of maize, there has been growing interest in recent decades in achieving greater precision in nitrogen (N) recommendations, for two main reasons: (1) the relatively high amounts of N required by the crop to reach potential yields result in substantial N applications (Ciampitti *et al*., 2013; Castellanos *et al*., 2019); and (2) the efficiency of N recovery by maize is relatively low, leading

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to N losses that increase production costs and environmental pollution, thereby contributing to climate change (Woli *et al*., 2016; Lu *et al*., 2019; Banger *et al*., 2020b).

Various approaches have been developed for generating N fertilization recommendations for maize. These differ in terms of the required information, the accuracy of the generated recommendations, and the associated time and cost (Morris *et al*., 2018). The primary approaches include: (1) considering the attainable yield of the crop, which depends on soil and climate conditions, as well as the production system (Sawyer *et al*., 2006; Morris *et al*., 2018); (2) conducting field experiments with increasing N rates to estimate a response function through regression analysis, followed by economic analyses to determine economic optimal N rates (EONR), and generating a recommendation for production systems under similar soil and climatic conditions (Sawyer *et al*., 2006; Morris *et al*., 2018); and (3) using soil and plant analysis to derive crop-specific recommendations at the individual farmer´s field level (Sela *et al*., 2016; Morris *et al*., 2018).

Not all approaches consider an economic analysis for generating maize N fertilization recommendations. When such analysis is incorporated, the economic criterion of maximizing net income per unit area (MNIS) is generally used. This is done in different ways, considering: (1) the crop yield achieved with increasing N rates or the same yield but subtracting the yield without N (Sawyer *et al*., 2006; Wang *et al*., 2020b); (2) fixed costs, with or without fixed production costs (Morris *et al*., 2018; Wang *et a*l., 2020b); and (3) net incomes, based on net income (NI) or yields and the subsequent calculation of NI (Morris *et al*., 2018; Wang *et al*., 2020b).

According to the economic theory, maximization of NI with the MNIS economic criterion is linked to maximization of the amount of N that is economically viable to use in production, which consequently implies the highest fertilization costs. In this sense, this economic criterion is most appropriate for use in contexts where there is sufficient capital availability and low risk, or where agricultural insurance is in place. The recovery of N from fertilizer in maize generally ranges between 35 and 75 %, with the remainder being lost through various pathways such as leaching, volatilization, denitrification, and surface runoff (Morris *et al*., 2018). This low applied N use efficiency (NUE) implies that higher N rates will result in greater losses (Sela *et al*., 2016; McLellan *et al*., 2018; Millar *et al*., 2018). Since the MNIS economic criterion generates higher EONR than other economic criteria, it also tends to result in greater N losses.

The MNIS economic criterion allows working with different input/output price ratios, which can fluctuate due to changes in the prices of inputs or products. For N, as expected, an increase in the N/maize (grain) price ratio (N/MPR) will reduce the EONR (Sawyer *et al*., 2006; Morris *et al.*, 2018). This reduction of EONR leads to decreases in the optimal economic yield (OEY) and NI, which, within certain limits, may not be significant (Banger *et al*., 2020a; Wang *et al*., 2020a). Conversely, lower EONRs imply higher NUEs and, therefore, reduced N losses (Millar *et al*., 2018; Wang *et al*., 2020b).

This study utilized data from field experiments on the response of maize to increasing

N rates, conducted under both rainfed and irrigated conditions in areas with varying yield potentials. This study aimed to determine in maize: (1) the economic optimal N rate, economic optimal yield, and N use efficiency using the current N/maize (grain) price ratio for attainable yield levels; and (2) the effect of an increase in the N/maize (grain) price ratio on the economic optimal N rate, economic optimal yield, net income, N use efficiency, and the loss of N.

MATERIALS AND METHODS

Data from 140 maize response experiments to increasing N rates were analyzed. The experiments were conducted by the International Maize and Wheat Improvement Center (CIMMYT) in 18 states in Mexico between 2010 and 2019, under both rainfed and irrigated conditions with the maize-maize production system. The states were grouped into distinct climatic regions. The prevalent agricultural soils included Phaeozems, Vertisols, Cambisols, and Luvisols, characterized mainly by medium and fine textures, and sandy soils in some states like Puebla and Tlaxcala (INEGI, 2007). The climate across these regions varied with latitude, altitude (ranging from 10 to 2250 m), relief (including coastal plains, mountainous areas, and high plateaus), and proximity to the coast (INEGI, 2018).

In the coastal plains, the climate is very warm, ranging from dry in the north to subhumid in the center and south of the country. In mountainous areas and high plateaus, the climate is temperate, subhumid, semidry, or dry, depending on their distance from the coast (INEGI, 2018). Rainy season occurs during the summer months, between June/July and September/October. The experiments were established: (1) at the onset of the rainy season (June/July) under rainfed conditions with supplemental irrigation when rains are scarce (spring-summer cycle); and (2) in October/November under irrigation in warmer areas with limited rainfall (autumn-winter cycle). The states, altitude, average annual rainfall, and number of experiments across rainfed and irrigated conditions were grouped by climatic region (Table 1).

Of the experiments, 107 were conducted in the spring-summer cycle and 33 in the autumn-winter cycle, with some experiments being replicated at the same sites in different years. The maize used included 131 experiments with improved varieties and nine with landraces. Five N rates were used in most experiments and, in some, seven rates, ranging from a treatment without N to 300 kg N ha^{-1} under rainfed conditions (based on the expected yield) and to 360 kg N ha^{-1} under irrigated conditions. The N fertilizer was urea, applied at sowing, as the experiments were specifically designed to estimate the optimal N rate for application at this time.

The experimental design was a randomized complete block, with each treatment replicated three or four times, subject to space availability. The size of the experimental plots was set at 5.6 x 8 m, including six rows with 0.8 m spacing between them. All experiments included a base phosphorous fertilization, with sowing, dates, seed doses, and the management of weeds, pests, and diseases carried out in accordance with the

Table 1. Altitude, average annual rainfall, and number of maize experiments under rainfed and irrigated conditions by climatic region.

Region	Altitude (m)	Average annual rainfall (mm)†	Number of experiments	
			Rainfed	Irrigated
Sinaloa, Sonora	25–50	250–450, 450–650	3	26
San Luis Potosi	100–150	800–1500	2	0
Guanajuato, Jalisco, Michoacan, Queretaro, Zacatecas	1300–1600, 1600–1900, 1900–2200	300–500, 500–700, 700–800, 800–1000	11	46
Hidalgo, Mexico, Puebla, Tlaxcala	2100–2500	300–500, 500–700, 700–1000	7	1
Morelos	900–1100	800–1000	10	1
Oaxaca	1500–2150	750	5	0
Chiapas, Guerrero	500–800	650–1050	15	2
Campeche, Quintana Roo, Yucatan	50–100	600–800, 800–1200, 1200–1400	11	0

†Source: INEGI (2018).

guidelines provided by the National Forestry, Agricultural and Livestock Research Institute (INIFAP). The response variable measured was grain yield at 14 % moisture. Data analysis for each experiment included a regression analysis to estimate a grain yield equation as a function of N rate, complemented by an economic analysis to calculate the EONR and EOY. The regression analysis included two key steps: (1) specifying models based on the observed graphical crop response to N; and (2) using the SAS (Statistical Analysis System) program to estimate the regression equation, eliminating possible outliers, when necessary ($p = 0.01$) (Freund and Littell, 2000), and selecting the model with the smallest mean square error as the most appropriate. With the fitted regression equation, EONR and EOY were determined using a SAS program designed for this purpose (Briones-Encinia *et al*., 2009).

The economic optimization criterion applied was the MNIS, where the EONR is determined at the point where the marginal net income (the increase in net income from an additional unit of input) equals the marginal cost (the cost of one unit of input). The price of fertilizer and maize was based on May 2020 figures. Given the prevalent use of urea and ammonium sulfate as N sources by farmers, the prices per kilogram of N for both fertilizers were averaged to USD \$1.06 N kg^{-1}, considering individual prices of USD \$0.955 and 1.165 N kg^{-1}, respectively. Transportation and application costs, as well as invested capital were added to this value, resulting in a price of USD \$1.25 N kg^{-1}. The net price of maize, after deducting harvest and transportation costs, was set at USD \$175 Mg^{-1}. With these prices, the N/MPR was equal to 7.14. All other costs related to crop production were considered fixed, amounting to USD \$425 and 575 ha^{-1} for rainfed and irrigated conditions, respectively.

The experiments were grouped based on moisture regime and attainable EOY into levels: (1) for rainfed maize, < 2, 2–3, 3–4.5, 4.5–6, 6–8, and 8–10 Mg ha^{-1}; and (2) for irrigated maize, 8–10, 10–12, 12–14, 14–16, and greater than 16 Mg ha^{-1}. For these attainable EOY levels, mean EONR, EOY, yields without N (Y_0), and NUE were determined. The NUE was estimated using the formula: NUE = ((EOY-Y_0) / EONR) x 20, where, the factor 20 represents the average amount of N (kg N) needed to produce 1 Mg of maize grain (14 % moisture) including stalk, assuming a harvest index of 0.5 for improved maize (Castellanos *et al*., 2019).

This approach does not account for the possibility that Y_0 may have a lower N concentration in the grain (Ciampitti and Vyn, 2014; Wang *et al*., 2020b), resulting in the NUE estimated being somewhat higher. With the N/MPR increased by 25, 50, 75, 100, 125, and 150 %, for maize experiments in both rainfed and irrigated conditions, EONRs were calculated similarly to the current N/MPR. Using the recalculated EONR, EOY, NI, and NUE were determined for these increased N/MPRs. Furthermore, based on the determined EONR, the potential N saving from using a lower rate was also estimated, thereby reducing both costs and environmental impact.

RESULTS AND DISCUSSION

Yields

The yields observed in the maize experiments, both with and without N application, show variations under rainfed and irrigated conditions. These differences can be attributed to the diverse soil types and climate conditions in which the experiments were established, and, when applicable, to the varying intensity of irrigation. The average yields and their standard deviations were: under rainfed conditions, 2.952 ± 2.136 Mg ha^{-1} (without N) and 4.971 ± 2.716 Mg ha^{-1} (with N); and under irrigated conditions, 5.816 ± 3.317 Mg ha^{-1} (without N) and 11.776 ± 2.366 Mg ha^{-1} (with N).

Models and fitting of regression equations by experiment

The regression equations from experiments differed in the number of N variables included, ranging from one to three, such as $N^{0.25}$, $N^{0.50}$, $N^{0.75}$, N, N^2, and N^3, depending on maize response to N. The most frequent models were square root (in 24 experiments) and quadratic (in 68 experiments). The fit of these regression equations, indicated by the R^2 value, ranged from 0.222 to 0.982, excluding experiments with no response to N. The correlation between the ordinate of the regression equations and the yield of the treatment without N was strong, with r = 0.998 ($p \leq 0.01$).

Economic optimal rate of nitrogen by level of attainable yield

The results show that in both moisture conditions (Table 2), an increase in Y_0, EOY, and EONR is followed by an attainable yield level increase, with a smaller increase in Y_0 under irrigated condition. The relationship tends: (1) to be linear, except for attainable

Table 2. Yields without nitrogen (Y_0), optimal economic yields (EOY), and economic optimal nitrogen rate (EONR) by level of attainable yield for rainfed and irrigated maize.

Moisture condition	Yield level (kg ha^{-1})	Yield (Mg ha^{-1})		EONR (kg N ha^{-1})
		Y_0	EOY	
Rainfed	< 2.0	1.020	1.150	9
	2.0–3.0	1.224	2.539	72
	3.0–4.5	1.881	3.768	99
	4.5–6.0	2.278	5.557	142
	6.0–8.0	3.129	6.888	157
	8.0–10.0†	5.199	8.978	154
Irrigated	8.0–10.0	4.838	9.356	187
	10.0–12.0	5.126	10.850	221
	12.0–14.0	5.451	13.381	269
	14.0–16.0	5.647	14.955	303

†The 8–10 kg ha^{-1} yield level in the rainfed condition displayed high without nitrogen and economically optimal yields, and for this, a relatively low economic optimal N rate, for which there is no response.

yield levels below 2 Mg ha^{-1} and from 8 to 10 Mg ha^{-1} under rainfed conditions that do not follow this trend; and (2) under irrigated conditions, this relationship follows a similar trend to rainfed conditions but with higher EONR and EOY values (Figure 1).

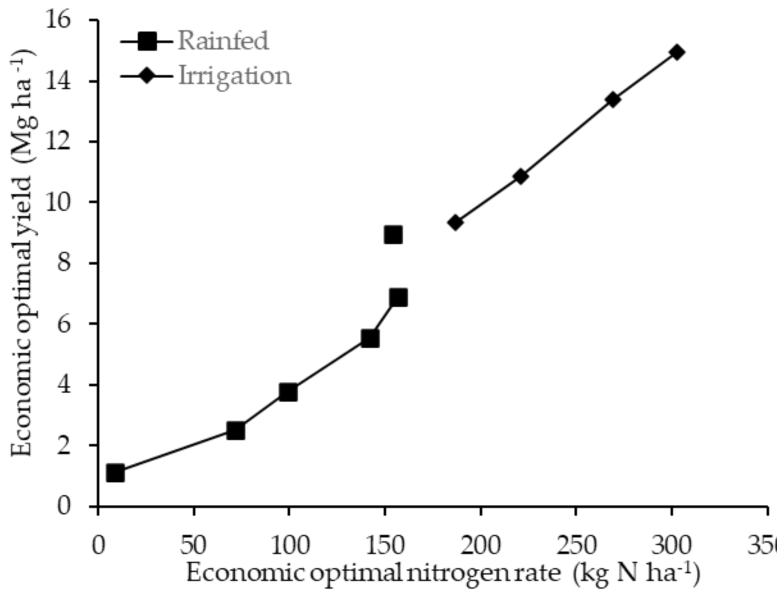

Figure 1. Relation between the economical optimal yield and the economic optimal nitrogen rate by level of attainable yield for rainfed and irrigated maize.

This study found that the relationship between EONR and EOY is similar to those reported by Sánchez-Roldán *et al*. (2022) in their study across 16 Mexican states, involving 67 experiments on maize response to N in soils with 2 % of organic matter. In the 67 experiments, N application was split into one-third at sowing and two-thirds at 30–45 days for expected yields below 4 Mg ha^{-1}, and all the N was applied at 45–60 days for expected yields above 4 Mg ha^{-1}. In the current 140 experiments, all N was applied at sowing. According to this, the time of N application would not have significantly influenced the response of maize to N.

It could be assumed that the maize response to N is mainly determined by yield and N supply from the soil. In 67 experiments, this N supply was estimated by soil organic matter content, and in 140 experiments, this was done by the yield of the treatment without N. The organic matter in the soil requires to be mineralized, and this process is influenced by soil and climate factors, as well as crop management (Wang *et al*., 2020b). In this case, crop management was referred to as the maize-maize system, but the soil types were different, as were the climate conditions.

In general terms, one way to reduce the effect of soil and climate variations on the crop response to N in a region is by mapping relative similar soils and climates in geographic areas (Sawyer *et al*., 2006; Morris *et al*., 2018). For climate variations, it is necessary to estimate the variation over the long term, excluding atypical years, or using a probabilistic distribution for more accuracy (Morris *et al*., 2018). In these geographic areas, experiments on the response of maize to N are carried out, and the average optimal rate of N are determined.

The EONR determined for maize yield levels can have the advantage that farmers know the yield their crop can achieve, unless it is limited by poor crop management. However, N recommendations determined for similar soil and climate units, as well as for stable management systems, may have some inherent lack of precision due to variations in soil N input by the production system (Wang *et al*., 2020b). These variations are due to differences in both the content and mineralization of soil organic matter, typical of any production system (Morris *et al*., 2018).

Relationship of the economic optimal nitrogen rate with the yields without nitrogen and the economic optimal yield

In order to deepen into the relationship between EONR against Y_0 and EOY (Table 2), regression equation for EONR was estimated as a function of Y_0 and EOY for both the rainfed and irrigated conditions. The estimated regression equations are as follows:

$$EONR_{rainfed} = 9.338 + 54.803\ Y_{os} - 2.201\ Y_{os}^2 - 61.906 Y_0 + 3.195\ Y_0 Y_{os}$$

(MSE = 154.115, R^2 = 0.979, n = 64, 5 outlier points removed),

$$EONR_{irrigation} = 44.301 + 48.776\ Y_{os} - 1.598\ Y_{os}^2 - 52.657 Y_0 + 0.533\ Y_{o0}^2 + 2.196\ Y_0 Y_{os}$$

(MSE = 321.131, R^2 = 0.971, n = 71, 7 outlier points removed), where EONR is the economic optimal nitrogen rate (kg N ha^{-1}), Y_{oe} is the economic optimal yield (Mg ha^{-1}), Y_0 is the yield without N (kg ha^{-1}), MSE is the mean square error, and R^2 is the coefficient of determination.

The regression equations have a good fit, expressed by high R^2 values for both rainfed and irrigated conditions, which could have been favored by the elimination of outlier points. These regression equations show: (1) that EONR increased with increasing EOY (positive decreasing relationship) and decreased with increasing Y_0 (negative relationship); and (2) a positive interaction between Y_0 and EOY, which indicates that the greater the EOY, the smaller the negative effect of Y_0.

The rise in EONR with yield level suggests that higher yields lead to more N being extracted from the fertilizer by the maize plants, with the amount of N extracted largely dependent on biomass production and its N concentration. This concentration depends on genetic factors (Ciampitti and Vyn, 2012) and can be modified by N contribution from the soil and N fertilization (Ciampitti and Vyn, 2014; Wang *et al*., 2020b), as well as by factors that limit crop yields (Morris *et al*., 2018). For improved maize with a harvest index of 0.5, concentration normally varies by about 20 kg of N for every 1 Mg of grain (plus stalk) (Castellanos *et al*., 2019).

In this study, the EONR displayed a correlation with yield level, suggesting that higher yields require more N, and therefore, a higher EONR might be necessary. There is information that corroborates this relationship (Morris *et al*., 2018; Wang *et al*., 2020b); however, it has also been observed that improved maize varieties with higher yields do not necessarily demand significantly more N from fertilizers, leading to a higher EONR. This is attributed to: (1) more developed root systems in newer maize generations that absorb N more efficiently, from both the soil and the fertilizer (Peng *et al*., 2010; Feng *et al*., 2016); and (2) the fact that the latest improved maize varieties produce more biomass per unit of absorbed N; therefore, a higher yield does not cause a proportional increase in the N demand (Ciampitti and Vyn, 2012; Woli *et al*., 2016).

On the other hand, the reduction in the EONR with an increase in Y_0 could be linked to a higher contribution of N from the soil due to a greater organic matter content derived from crop residues and root production. This was observed in the study, where the correlation between Y_0 and EOY was r = 0.691 ($p \leq 0.01$) in rainfed experiments and r = 0.402 ($p \leq 0.01$) in irrigated experiments. However, the residual N content in the soil may also be related to this (Wortmann *et al*., 2011; Morris *et al*., 2018).

The attainable yield, represented here by the EOY, has been the basis of the model for generation N recommendations in maize, as utilized in many states in the USA between 1970 and 2005 (Morris *et al*., 2018). This model, however, has been questioned for overlooking economic considerations in its recommendations and for the attainable yield not being related to the EONRs (Sawyer *et al*., 2006; Morris *et al*., 2018). In this latter point, it is worth considering the relationship to consider the relationship between EOY and EONR alongside the N contribution from the soil, estimated in this study by Y_0. Thus, a similar yield might be achieved with either a higher N contribution from the soil and a lower EONR or a lower N contribution from the soil and a higher EONR. This was substantiated by the regression analysis, where the EOY as a function of EONR showed R^2 values of 0.334 ($p \leq 0.01$) for experiments under rainfed conditions and 0.002 ($p \leq 0.74$) for experiments under irrigated conditions. However, when incorporating both EONR and Y_0, the R^2 values increased to 0.93 ($p \leq 0.01$) and 0.676 ($p \leq 0.01$), respectively.

Applied nitrogen use efficiency

In addition to the crop, the applied N use efficiency (NUE) is influenced by several factors, including soil characteristics (mainly texture), climate (the frequency and distribution of rainfall, and temperature), and the management of fertilization (its chemical form, application method, timing, and the amount applied relative to the soil N contribution). These factors contribute to variations in NUE both within and between production systems (Morris *et al*., 2018). For diverse conditions soil, climate and N fertilizer management, mean NUE values between 0.35 and 0.75 are reported for maize (Morris *et al*., 2018), and as high as 0.85 in maize under optimal irrigation conditions (Castellanos *et al*., 2019).

In this study, the NUE for EONR increased with the level of attainable yield in both moisture conditions, as values: (1) under rainfed, 0.29, 0.37, 0.38, 0.46, 0.48, and 0.49 for yield levels < 2, 2–3, 3–4.5, 4.5–6, 6–8, and 8–10 Mg ha^{-1}, respectively; and (2) under irrigated, 0.48, 0.52, 0.59, and 0.61 for yield levels 8–10, 10–12, 12–14, and 14–16 Mg ha^{-1}, respectively. These estimated efficiencies are relatively low in comparison with values obtained with split N applications in other studies. For example, in irrigated maize, Wortmann *et al*. (2011) report an average NUE of 0.67 for a mean yield of 13.3 Mg ha^{-1} obtained with a mean rate of 171 kg N ha^{-1}, and Davies *et al*. (2020) report a value of 0.63 for a yield of 13.7 Mg ha^{-1} obtained with a rate of 202 kg N ha^{-1}. In rainfed maize: (1) Wang *et al*. (2020b) report mean NUEs between 0.56 and 0.58 for mean yields between 10.45 and 10.34 Mg ha^{-1} obtained with rates of 225 and 187 kg N ha^{-1}, respectively; and (2) Meena *et al*. (2020) report a NUE of 0.36 and 0.39 for yields of 3.88 and 4.66 Mg ha^{-1} obtained with rates of 90 and 120 kg N ha^{-1}, respectively, which are similar values to those found in this study. However, the NUEs estimated in this study were obtained by applying N at sowing, which could partially explain the relatively low NUE values reached.

In order to deepen into the relationship between NUE vs. EONR and EOY, regression equations of the NUE were estimated as a function of such variables for both the rainfed and irrigated conditions. The estimated regression equations are as follows:

$$NUE_{rainfed} = 0.216 + 0.167\ Y_{os} - 0.148\ Y_0 - 0.000932\ N_{oe} - 0.000323\ Y_{oe} N_{oe} + 0.000174\ Y_0 N_{oe}$$

(MSE = 0.00322, R^2 = 0.778, n = 43, 6 outlier points removed),

$$NUE_{irrigation} = 0.208 + 0.123\ Y_{os} - 0.108\ Y_0 - 0.000906\ N_{oe} - 0.000155\ Y_{oe} N_{oe} + 0.000889\ Y_0 N_{oe}$$

(MSE = 0.00578, R^2 = 0.960, n=60, 4 outlier points removed), where NUE is the nitrogen use efficiency, Y_{oe} is the economic optimal yield (kg ha^{-1}), Y_0 is the yield without N (kg ha^{-1}), N_{oe} is the economic optimal nitrogen rate (kg N ha^{-1}), MSE is the mean square error, and R^2 is the coefficient of determination.

Both regression equations show that NUE increased with increasing EOY and decreased with increasing EONR and Y_0. Also, EOY has a negative interaction with EONR, and Y_0 has a positive interaction with EONR. The increase in NUE due to higher EOY coincides with greater biomass production by the plants, leading to an efficient root system that can utilize applied N more efficiently (Peng *et al*., 2010; Feng *et al*., 2016). Conversely, as the rate of applied N increases, its relative use efficiency tends to decrease, as indicated by numerous studies on the matter (Wortmann *et al*., 2011; Millar *et al.,* 2018).

Economic optimal nitrogen rate obtained with increased nitrogen/maize price ratios

The N/maize price ratio (N/MPR) has been utilized to determine the EONR based on maximizing economic income, considering variations in the prices of N and maize (Dobermann *et al*., 2011; Morris *et al*., 2018). In this study, EONRs were calculated using regression equations from experiments, considering both the current N/MPR and increased N/MPR by 25, 50, 75, 100, 125, and 150 %. The analysis was applied to 19 experiments with yield ranges between 5 and 8.5 Mg ha^{-1} under rainfed conditions and 29 experiments with yield ranges between 10 and 14 Mg ha^{-1} under irrigated conditions. The resulting EONRs were then used to determine EOY, cost of N, and NI for respective attainable yield levels, and an average value was estimated for each N/MNR (Table 3).

An increase in N/MPR led to a decrease in the related EONR and costs of N fertilization, as well as reductions in EOY and NI (Table 4). However, the percentage reductions in EONRs and their associated costs were more significant than the percentage reductions in EOYs and NIs. For instance, with a 100 % increase in N/MPR: (1) under rainfed conditions, EONR was reduced by 38 kg N ha^{-1} (21.5 %) and its cost by USD \$47.5 ha^{-1} (21.5 %), compared to a decrease of 0.397 Mg ha^{-1} (5.8 %) in EOY and USD \$21.95 ha^{-1} (4 %) in NI; and (2) under irrigation conditions, EONR was reduced by 45 kg N ha^{-1} (18.8 %) and its cost by USD \$56.25 ha^{-1} (18.8 %), compared to a decrease of 0.475 Mg ha^{-1} (4 %) in EOY and USD \$26.85 ha^{-1} (2.5 %) in NI. These reductions in EONRs with increased N/MPR have both economic and environmental implications. Economically, they decrease the cost of N fertilization without significantly reducing NIs, within certain limits. Environmentally, they contribute to reducing N losses by applying lower amounts of N to crops, thus mitigating environmental contamination. Practically, it is necessary to consider how much the N/MNR can be increased without significantly affecting yields and net incomes.

The higher the rate of N applied to the crop, the greater the potential losses through processes such as leaching, volatilization, denitrification, and surface runoff (McLellan

Table 3. Economic optimal nitrogen rate (EONR), economic optimal yield (EOY), cost of nitrogen (N), and net income (NI) from an increase in the N/maize price ratio (N/MPR) in maize under rainfed and irrigated conditions.

Moisture condition	Increase in N/MPR (%)	EONR (kg N ha^{-1})	EOY (Mg ha^{-1})	Cost of N (USD $ ha^{-1})	NI (USD $ ha^{-1})[§]
Rainfed[†]	0	177	6.861	221.25	554.40
	25	167	6.777	208.75	552.20
	50	157	6.687	198.75	548.95
	75	148	6.574	185.00	540.45
	100	139	6.464	173.75	532.45
	125	131	6.337	163.75	520.20
	150	122	6.172	152.50	502.60
Irrigated[¶]	0	240	11.771	300.00	1184.90
	25	229	11.681	286.25	1182.90
	50	217	11.568	271.25	1178.15
	75	206	11.437	257.50	1168.95
	100	195	11.296	243.75	1158.05
	125	185	11.133	231.25	1142.00
	150	175	10.967	218.75	1125.45

[†]Yield between 5 and 8.5 Mg kg ha^{-1}. [¶]Yield between 10 and 14 Mg ha^{-1}. [§]Fixed costs, USD $425 for rainfed, USD $575 for irrigation.

Table 4. Reduction of the economic optimal nitrogen rate (EONR), economic optimal yield (EOY), cost of nitrogen (N), and net income (NI) due to the increase in the N/maize price ratio (N/MPR) in rainfed and irrigated maize.

Moisture condition	Increase in N/MPR (%)	EONR (kg N ha^{-1})	EOY (Mg ha^{-1})	Cost of N (USD $ ha^{-1})	NI (USD $ ha^{-1})
Rainfed	0	0	0.000	0	0
	25	10	0.084	12.5	2.2
	50	20	0.174	50.0	5.4
	75	29	0.287	36.2	13.9
	100	38	0.397	47.5	21.9
	125	46	0.524	57.5	34.7
	150	55	0.689	68.7	51.8
Irrigated	0	0	0.000	0	0
	25	11	0.090	13.7	2.0
	50	23	0.203	28.7	6.7
	75	34	0.334	42.5	15.9
	100	45	0.475	56.2	26.9
	125	55	0.638	68.7	42.9
	150	65	0.804	87.2	59.4

et al., 2018; Millar *et al*., 2018). In this study, N losses were reduced based on economic considerations with the increase in N/MPR. However, strategies to reduce N losses and practices related to the use and management of N fertilizers should also be considered. Depending on the crop, soil, and climate, N losses are related to the management of the N fertilizer in relation to its chemical form, application time, application method, and the level of N rate relative to the soil N contribution (Morris *et al*., 2018; Banger *et al*., 2020b; Wang *et al*., 2020a). In addition, the use of soil and plant analysis leads to more accurate N recommendations, thereby reducing N losses (Morris *et al*., 2018).
For example, in the United States and China, which are leading producers of maize, there have been issues with N management. In 2011, in the USA, 71 % of drained lands did not have proper management in terms of rates, application time, and application methods (Ribaudo *et al*., 2011). Also, considering that de N fertilizer should be applied when the maize requires it: (1) in 2012, 91 % of farmers in Minnesota, USA, applied N in a single application (either in autumn or spring pre-planting) (Bierman *et al*., 2012), and (2) in China, in 2019, 51.4 % of farmers applied N in a single application to average rates of 248 kg N ha^{-1}, achieving mean yields between 9.1 and 10.9 Mg ha^{-1} (Chen *et al*., 2019).

The practice of applying excessive amounts of N to crops, particularly maize, is prevalent in several countries (Huang *et al.*, 2012; Ribaudo, 2015; Flores-Sánchez *et al*., 2019). The change to practices that reduce N losses is a complex process that covers not only economic and environmental aspects but also social ones. In this regard, there may be several factors and beliefs among farmers: (1) higher N rates would produce higher yields without considering that the relationship between both variables is not linear (positive, decreasing) (Chen *et al*., 2019); (2) farmers who have previously achieved satisfactory results applying high N rates are likely to continue using such rates (Sawyer *et al*., 2006); (3) farmers primarily focused on maximizing income, often at the expense of environmental considerations (Ribaudo, 2015); and (4) adopting improved technologies that increase NUE can be complex and difficult to adopt (Chen *et al*., 2019).

Various programs aimed at making N use more efficient in crops have been primarily voluntary, focusing on encouraging farmers to adopt practices that reduce N losses and, with some exceptions, have had limited impact (Ribaudo, 2015; McLellan *et al*., 2018). An alternative approach could be that farmers from support programs (such as agricultural insurance, services, and subsidies) should comply with recommended practices for management of N fertilizers (Ribaudo, 2015).

Increase in the nitrogen/maize price ratio and applied nitrogen use efficiency

The losses of N applied to crops are often indirectly inferred through NUE measurements, where higher NUE indicates lower N losses (Wortmann *et al.*, 2011; Millar *et al*., 2018; Lu *et al*., 2019). In the experiments discussed earlier, under both rainfed and irrigated conditions, NUE was calculated with increased N/MPR values (Table 5). As the EONR decreased due to the increase in N/MPR, the NUE correspondingly increased. For instance, with the N/MNR increasing from 0 to 150 %,

Table 5. Nitrogen use efficiency based on the increase in the nitrogen/maize price ratio in rainfed and irrigated maize.

Moisture condition	Increased nitrogen/maize price ratio (%)						
	0	25	50	75	100	125	150
Rainfed	0.47	0.49	0.51	0.54	0.56	0.58	0.61
Irrigated	0.53	0.55	0.58	0.60	0.62	0.63	0.67

the NUE increased from 0.47 to 0.61 under rainfed conditions and from 0.53 to 0.67 under irrigated conditions (Table 5). Therefore, utilizing the N/MNR in line with the MNIS economic criterion not only leads to a reduction in EONR but also an increase in NUE, thereby reducing N losses. (Table 4).

CONCLUSIONS

Based on maize yield data obtained from experiments with increasing N rates under diverse climate and soil conditions in Mexico, economic optimal N rates (EONR) were determined for attainable maize yield levels under rainfed and irrigated conditions, which may have practical applications at a regional level. An increase in the N/maize (grain) price ratio (N/MPR) led to a decrease in the economic optimal N rates (EONR), resulting in lowered costs of N fertilization without significantly impacting yields and net incomes (NI). The N use efficiency (NUE) increased with: (1) the rise in the level of economic optimal yield (EOY); and (2) the decrease in the economic optimal N rate related to an increasing N/maize (grain) price ratio. The use of N/maize (grain) price ratios was a way to reduce both the economic optimal N rates and, consequently, the N losses, thereby mitigating environmental pollution.

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WHAT DRIVES FARMERS TO ADOPT PRO-ENVIRONMENTAL BEHAVIOR? EVIDENCE FROM DATE FARMS IN SAUDI ARABIA

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ABSTRACT

Despite widespread knowledge of agriculture's sustainability challenges, more needs to be known about the factors influencing farmers' adoption of sustainable practices. This study examines the specific drivers of pro-environmental behavior among date farmers. A variable model was developed, and a questionnaire was used to gather farmers' perceptions of the factors influencing pro-environmental behavior on date farms. A hierarchical structure of the identified factors was developed using MICMAC analysis on 81 farmers in the kingdom of Saudi Arabia. Our results show that farm size, supply chain pressure, and firm technological capabilities are the most relevant determinants of pro-environmental behavior on date farms. Also, farmers' experiences, learning from other firms in the sustainability domain, and farm image improvement are critical variables in the system of pro-environmental behavior adoption. Finally, we argue that pressure from environmental regulation and the farmers' age are excluded variables. It is crucial to consider that relying solely on encouragement and laws to protect the environment cannot achieve sustainability in agriculture. More attention should be devoted to educating farmers about the importance of pro-environmental behavior and how it can improve their image in front of stakeholders. It is also necessary to organize periodic forums to support mimetic pressure and push farmers to learn from each other. Because farmers' environments in developing countries tend to be similar, it is possible to generalize these results to some extent. This model unveils a fresh perspective on promoting sustainability in agriculture by using novel analytical techniques to map relationships between pro-environmental actions and their influencing factors.

Keywords: new institutional theory, legitimacy theory, human capital theory, cognitive mapping.

INTRODUCTION

In recent years, attention has been clearly directed to environmental preservation and sustainability due to climate change, high levels of pollution, and the emergence of many epidemics and pests (Halaburda and Bernaciak, 2020). In particular, pro-environmental behavior, which represents a critical factor in reaching sustainability in various economic activities, has developed as a new term, especially in agriculture. Although there are several studies on the motives that determine the pro-environmental behavior of farmers, there is no explicit agreement about them. As a result, more research is needed, particularly in agricultural areas where farmer behavior and commitment to environmental conservation are directly affected.

This study seeks to determine the factors influencing date farmers' adoption of pro-environmental behavior in the Qassim region in Saudi Arabia. This region is considered the largest producer of dates in the Kingdom and one of the largest suppliers of several types of dates to the global market. The research problem of this study is considered extremely important, especially in light of the Kingdom of Saudi Arabia's Vision 2030, which aims to create a robust agricultural sector that achieves the anticipated sustainability goals and includes agriculture as one of its most crucial pillars. To protect the environment, it is essential to assess how well farmers adhere to the fundamentals of a sustainable lifestyle (Ataei *et al*., 2018). Agriculture is a significant source of income in developing nations. As a result, environmental changes have significant social and economic repercussions (Yaghoubi Farani *et al*., 2021). In order to develop a clean production policy and maintain pro-environmental behavior, this study seeks to identify the most significant factors influencing farmers' behavior on date farms. Dates are one of the nation's key products since palm trees can survive in arid climates, and the Kingdom of Saudi Arabia is one of the greatest palm tree growers.

It is crucial to use environmentally friendly practices in order to achieve sustainability. Long-term management techniques and the adoption of modern agricultural practices are required to guarantee the survival of palm trees under current conditions and future climate scenarios (Elhendy and Al Katani, 2013). The sustainability of date agriculture is dependent on farmers implementing sustainable agricultural practices. Their decision-making is influenced by beliefs, conventions, experiences, values, tribal patterns, educational attainment, and age (Mobeen *et al*., 2016). Therefore, we identified the factors that drive date farmers to adopt pro-environmental behavior and make sustainable decisions. For this, a theoretical framework based on three theories that are most relevant to the issue of pro-environmental behavior was used. Specifically, we relied on the new institutional, legitimacy, and human capital theories.

The new institutional theory suggests that the behavior of actors within an organization or system is influenced by the institutional environment in which they operate. This means that farmers' behavior is not only influenced by their own individual preferences and beliefs but also by the broader institutional context in which they operate, including social norms, values, and regulations. Legitimacy theory suggests that organizations, including farms, must maintain legitimacy in the eyes of stakeholders in order to survive and thrive. Farmers must engage in socially responsible and environmentally sustainable practices in order to maintain legitimacy with consumers, regulators, and other stakeholders. Human capital theory suggests that investments in education, training, and experience can enhance an individual's productivity and contribute to their success. This can be applied to farmers who invest in sustainable agriculture practices, which can increase their productivity and profitability over the long term. These theories help us understand how farmers make decisions on sustainable agriculture practices and how they can be encouraged to adopt more pro-environmental behaviors over the long term.

Previous studies indicate that there are 13 factors that are likely to influence their behavior, derived from the use of the three theories previously discussed. Based on a review of studies that fall under the new institutional theory, five hypotheses were developed. First, customer pressures can affect the farmers' pro-environmental behavior. Tey *et al*. (2014) mentioned that certain customer requirements have to be satisfied in agriculture. Second, the pressure of government regulations has a positive effect on farmers' pro-environmental behaviors. Regulation-related restrictions all have a considerably positive effect on farmers' decisions for organic fertilizer and pro-environmental behavior adoption (Lv *et al*., 2023). According to Li *et al*. (2022), environmental regulations and risk perception both significantly affect farmers' desire to adopt sustainable practices. These pressures are related to competitive relations between farmers and agriculture organizations.

Third, we assume that pressure from environmental organizations has a positive effect on farmers' pro-behaviors. Fu *et al*. (2017) found that group pressure significantly influences the development of individual environmental behaviors. Other studies have affirmed the existence of a positive relationship between pro-environmental behavior and group pressure, such as Banwo and Du (2019). Our fourth hypothesis assumes that learning from others pressure has a positive effect on farmers' behaviors toward agriculture businesses. Thus, it is considered an important factor and helps them follow clean production policies. According to Juárez-Luis *et al*. (2018), pressures have the biggest influence on green practices. Learning from other experiences is primordial in this case (Atanasovska *et al*., 2022). Tey *et al*. (2014) affirm that learning from other firms could influence their behaviors, especially when they know each other and share a common language.

Our fifth hypothesis stipulates that supply chain pressure positively influences farmers to adopt pro-environmental behaviors. Organizations and farms should encourage their employees to become environmentally aware by engaging them in pro-environmental behaviors. Paillé and Boiral (2013) stated that there is growing attention among researchers to find the backgrounds of employees' pro-environmental behaviors at work. In order to satisfy the needs of customers, regulators, and other stakeholders, farmers may be encouraged to adopt more sustainable practices throughout their supply chains.

This study also relied on the legitimacy theory, which implies that the farmer considers polishing his reputation and improving his image in relation to the categories of stakeholders. This is what drives them to adopt pro-environmental behavior in all their decisions and activities. In this regard, Vilkė *et al*. (2021) found that farmers' awareness of environmental responsibility in terms of eco-efficiency and clean production has relevant implications. Farmers may experience negative social or economic effects if they are known in their community for indulging in unsustainable or environmentally harmful practices. They may be discouraged from engaging in unsustainable activities as a result. Farmers are more prone to group pressure, desire to have a good reputation in the group, and anticipate having neighbors' trust (Castro-Campos, 2022). Generally, reputation and image are strong inducers of pro-environmental behavior and can set up a positive feedback loop in which the adoption of sustainable techniques is reinforced by the enhancement of the farmer's reputation.

The effect of farm size on sustainable supply chain management and performance has received little research. Because larger businesses have more resources at their disposal and are subject to more environmental pressure than smaller businesses, company size may have an impact on how well environmental practices are implemented (Wang *et al*., 2023). According to Ren *et al*. (2019), farm size is linked to a statistically significant drop in the amount of fertilizer and pesticides used per hectare. The use of herbicides and pesticides would generally decline by 1.8 % and the use of fertilizers by 0.3 % for every 1 % increase in farm size.

Using previous studies, we developed three hypotheses using this theory as potential determinants of farmers' pro-environmental behavior. The first one stipulates that the engagement of farmers in corporate social responsibility has a positive impact on farmers' pro-environmental behaviors. The second predicts that corporate farm image improvement will positively influence farmers to adopt pro-environmental behaviors. Finally, the third hypothesis related to legitimacy theory predicts that farm size influences farmers positively to adopt pro-environmental behaviors.

On the other hand, human capital theory suggests that there are significant factors that can influence farmers' behavior and their willingness to adopt pro-environmental behavior. Based on this theory, we hypothesized that the farmer's age, time of experience in their field of activity, and gender can largely affect their behavior. Fang *et al.* (2018) found that younger people are more concerned about the environment than older adults. Recent review articles by Glazebrook *et al*. (2020) provide a strong foundation regarding the relationship between gender and environmental concerns in agriculture. Farmers with more experience should be more knowledgeable and proficient when assessing and interpreting information. We concur that farmers with experience are more likely to implement agriculturally pro-environmental practices (Chizallet *et al*., 2023).

Finally, the current study assumes that there are two additional variables that could have explanatory power for farmers' adoption of pro-environmental behavior, which are the technological capability of the farm and the desire to reduce costs. In fact, farms will require the first to adopt pro-environmental technologies, as well as the ability to create projects to execute and modify their processes, operations systems, and information systems (Oke, 2023). Regarding cost savings, Savari *et al*. (2021) confirm that the familiarity of farmers with the benefits of pro-environmental behavior adoption can lead to their increased positive attitude towards pro-environmental behavior, which can directly lead to increasing crop yields. Thus, it is important to accelerate the transformation of agricultural production methods, dynamically develop green agriculture, and protect the environment while increasing incomes (Liu *et al*., 2023).

MATERIALS AND METHODS

The MICMAC analysis technique

In order to determine the most influential factors on the pro-environmental behavior of date farmers, we relied on mind maps using the MICMAC software. In fact, this technique aims to discover how date farmers view the issue of pro-environmental behavior and what factors they see as actually influencing this responsible behavior. If we identify the factors that are likely to influence farmers' behavior and make them adopt sustainable behavior, it is actually easier to direct their behavior in this area.

The structural analysis method was used, specifically the MICMAC method. First, the variables likely to affect the studied phenomenon are identified. Secondly, the adjacency matrix is filled. Third, an adjacency matrix must be entered into the MICMAC software, and the analysis must be performed via the matrix of direct influence and the matrix of indirect influence (Arcade *et al*., 1999). After constructing an individual cognitive map for each farmer, we combined these individual cognitive maps to obtain the aggregated cognitive map, which summarizes farmers' perceptions around the critical determinants of pro-environmental behavior. We applied the arithmetic mean to go from individual to aggregated cognitive maps (Lajnef *et al*., 2017).

A battery of analysis tools was applied to analyze farmers' perceptions. The centrality analysis was used to identify the most critical factors that can influence the pro-environmental behavior. The MICMAC software can easily generate a variable ranking based on the capacity of each variable or concept to influence other concepts in the whole system and its dependence degree in the studied phenomenon. We focused on the centrality analysis, which supposes that in a given system, the most central concept has the highest number of entry and exit arcs. For this, we concentrated on the aggregated cognitive map generated. We used the influences-dependences chart since it can give a clear idea of how all proposed variables as potential determinants of farmers' pro-environmental behavior are clustered in farmers' mental schemes.

The influences-dependences chart (Figure 1) generated by the MICMAC software comprises four quadrants. The first represents the input/influential variables that seem to significantly impact farmers' pro-environmental behavior adoption (Chatziioannou

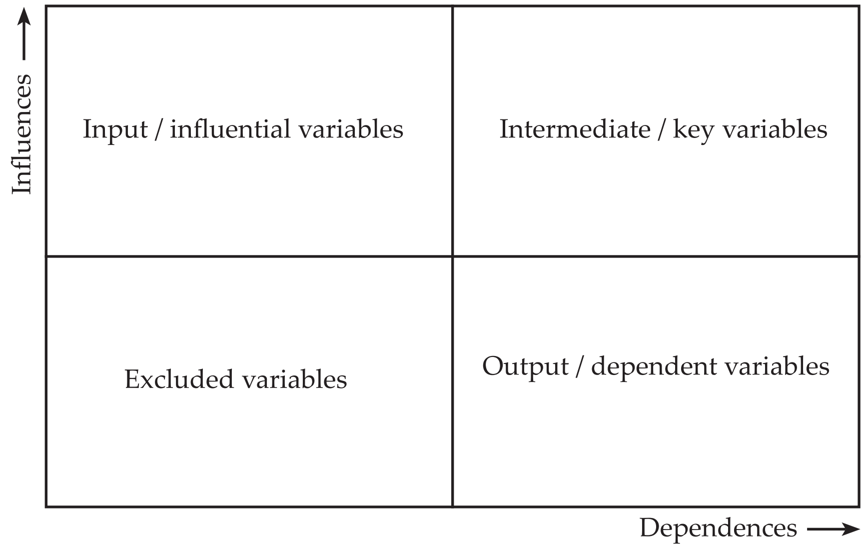

Figure 1. Influences-dependences chart showing the general explanatory value arrangement of the variables.

et al., 2023). Furthermore, these variables are the most influential and can have an explanatory power for the studied phenomenon. The second quadrant contains the relay variables, also called intermediate/key variables due to their ability to influence the whole system (Ben Fatma *et al*., 2021). The third quadrant shows the dependent variables with low influence and strong dependence which have low explanatory power. Finally, the fourth quadrant contains excluded variables that represent variables with no explanatory power, and so are rejected by the system (Chatziioannou *et al*., 2023).

Study area description

This research was carried out in the Qassim region of the Kingdom of Saudi Arabia. It is located approximately 400 km northwest of Riyadh. Al-Qassim is known as the alimental basket of the country due to its distinction in agriculture, especially the production of different types of dates. Data was collected in approximately four months, from January to April 2023. According to official statistics, the Qassim region produces 200 thousand Mg of dates annually (Ben Mohamed *et al*., 2024). This makes the subject of the study particularly important, given the findings of some published studies by Abdallah *et al*. (2018), where the presence of pesticide residues was proven in 18 % of the sample they examined.

Sample collection

The self-structural interaction matrix was produced based on previous studies in the field of pro-environmental behavior. A set of questions was developed to collect basic data about the farmers who participated in the survey, such as age and experience. Before distribution, the questionnaire was presented to a small group of three farmers to ensure its clarity. Then, it was sent to the Ethics Subcommittee and approved. The purpose of the questionnaire was to make it easier to gather information in order to create the SSIM matrix.

The questionnaire was given to a total of 123 date farmers selected at random. At the end, 81 completed replies were obtained. The farmers interviewed were invited to estimate, for each variable and following our theoretical study and exploration of the field, the influence effects by filling out a structural analysis matrix. This matrix contained all potential determinants of pro-environmental behaviors, and farmers were asked to assign the impact of each variable on other variables. If the relationship is strong between two variables, then a value of 3 is assigned. If this relationship is medium, a value of 2 is assigned. If this relationship is weak, its value is 1. If there is no impact between variables, its value is 0.

RESULTS

The sample was heterogeneous in terms of the gender. It was composed of 87.6 % men and 12.4 % women (Table 1). All age groups were present. This is important to study the phenomenon considering all age groups' perceptions. Our sample contained farmers with different education levels and experiences. The questionnaire was distributed to farmers and was designed in a way that enables us to collect general information about the owner of each farm.

By looking at the aggregated adjacency matrix, we can extract the most critical variables central to pro-environmental behavior (Table 2) using the total influence score. The

Table 1. Sample descriptive statistics (n = 81) showing results from the respondents' general information.

Variables	Class	Frequency	Percentage
Age	≤ 30	11	0.135
	31–40	19	0.235
	41–50	18	0.222
	51–60	25	0.308
	> 60	8	0.098
Gender	Male	71	0.876
	Fiemale	10	0.124
Education level	No formal education	17	0.209
	Primary	20	0.247
	Secondary	18	0.222
	Tertiary	26	0.321
Years of experience	≤ 5	24	0.296
	5–10	16	0.197
	11–15	18	0.222
	> 15	23	0.284

Table 2. Adjacency matrix with influence scores assigned to all variables according to their potential impact on each other.

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	Total influences	Total influences and dependences
V1	0	2	1	2	1	1	2	1	2	3	2	2	2	21	44
V2	2	0	2	1	1	1	2	3	2	3	2	3	2	24	48
V3	2	2	0	1	1	1	2	1	2	3	2	2	2	21	42
V4	2	2	1	0	2	1	2	2	2	3	2	3	1	23	44
V5	1	1	1	2	0	2	3	2	2	2	2	3	2	23	44
V6	1	1	1	1	2	0	2	1	2	2	2	1	1	17	35
V7	2	2	2	3	2	2	0	2	2	2	2	3	2	26	53
V8	2	3	2	2	2	1	3	0	1	2	2	2	2	24	45
V9	2	2	2	2	2	2	2	1	0	2	2	2	2	23	47
V10	3	3	3	2	2	2	2	2	3	0	2	3	2	29	59
V11	2	2	2	2	2	2	2	2	2	3	0	2	1	24	48
V12	2	2	2	2	2	2	3	2	2	3	2	0	2	26	54
V13	2	2	2	1	2	1	2	2	2	2	2	2	0	22	43
Total dependences	23	24	21	21	21	18	27	21	24	30	24	28	21		

V_1: pressure of environment regulation; V_2: cost savings; V_3: customer pressure; V_4: farms technological capability; V_5: age; V_6: gender; V_7: experience; V_8: farm size; V_9: corporate social responsibility; V_{10}: corporate image improvement; V_{11}: supply chain pressure; V_{12}: learning from other farms; V_{13}: pressure from environmental organizations.

importance of each variable in the system can be noticed as it measures the extent of the variable's susceptibility to other variables in the system.

Depending on the extent of the variable's effect on the phenomenon of pro-environmental behavior, we find three central variables: corporate image improvement, farmer experience, and the ability to learn from other farms. Especially corporate image improvement, as predicted by the legitimacy theory, can be considered a central variable in the determinants of pro-environmental behavior. This variable has a total score of influences equal to 29, meaning it significantly influences other variables in this system. On the other hand, other studies consider gender to be an essential factor, which may explain why environmentally friendly production policies and behaviors are adopted by certain people. It appears that gender is the weakest variable in terms of its ability to influence the rest of the other variables in the system, according to what farmers see in the study sample, with a score equal to 17.

If we rely on the total influence score to arrange the variables according to their importance, we find that the results are almost constant, especially for the three central variables. Specifically, corporate image improvement is the most affected variable as it receives influences from various other variables in the system, with a total value of 30, followed by the variable of learning from other farms with a score equal to

28. Then, the farmer's experience is greatly affected by the rest of the variables in the system, with a score equal to 27. We also note that three variables are classified as fourth: corporate social responsibility, supply chain pressure, and cost savings. Finally, when taking into account the extent to which a variable can influence and be affected by the rest of the variables, we note that the central variables are: corporate image improvement, learning from other farms, experience, cost savings, supply chain pressure, and corporate social responsibility.

From the aggregated cognitive map (Figure 2), it is possible to notice several influence relationships among the variables that have been proposed to explain the motivation to adopt pro-environmental behavior. The map is overlapping, and several influence relationships exist between variables. These explain that the studied phenomenon has an actual impact on guiding the behavior of farmers in the study sample.

The map shows that the desire to maintain the good reputation, as predicted by the theory of legitimacy, is considered a significant variable, as it affects the majority of other variables in the map and is also affected by them. This indicates the importance of the interpretation proposed by the theory of legitimacy that farmers, in their attempt to build and maintain a good reputation, will adopt pro-environmental behavior to achieve this goal. In this regard, corporate image improvement directs the company towards pressure on costs and the extent of reliance on advanced and environmentally friendly technologies. It also has a considerable impact on the decision to adopt social responsibility and learn from other farms in the field of sustainability.

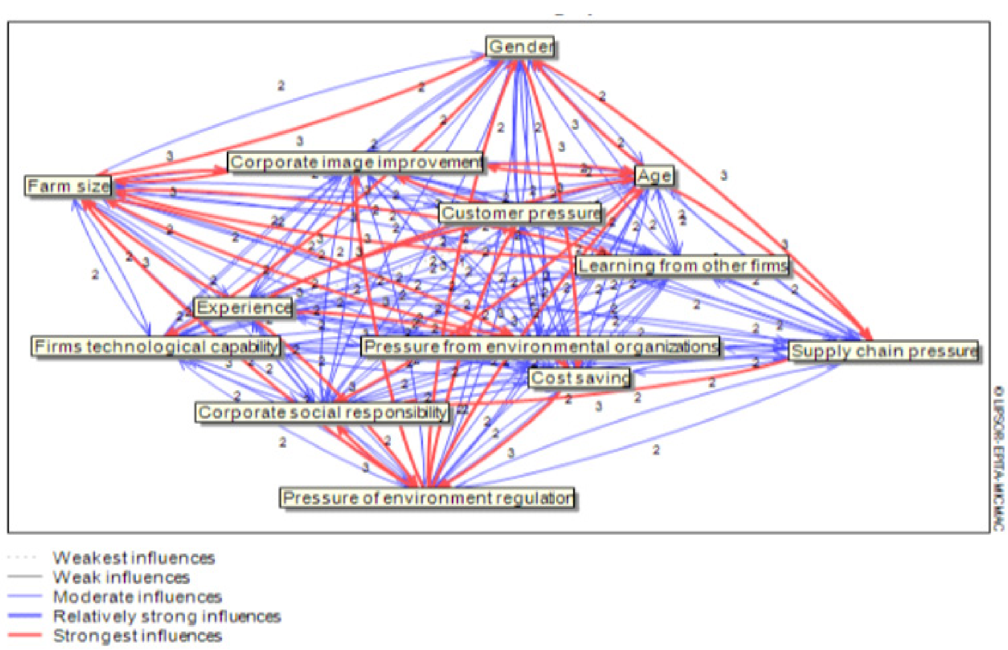

Figure 2. Aggregated cognitive map from direct influences of the variables influencing pro-environmental behavior.

One of the analysis tools that the MICMAC program can provide is the study of indirect relationships. The relationship between the variables goes beyond the limits of direct influence, as there are generally strong relationships between most of the study's variables, except for gender (Figure 3). There is still a robust relationship between creating and maintaining a good reputation for the farm and the ability of the farmer to learn from the experiences of other farms in the field of sustainability. It also appears that the farmer's experience has a strong indirect effect on the majority of the variables in the system, making it a significant variable in the farmers' mental map. Finally, it appears that gender does not significantly affect the rest of the variables that may affect farmers' decisions to adopt pro-environmental behavior. The effect of the rest of the variables on it is also weak.

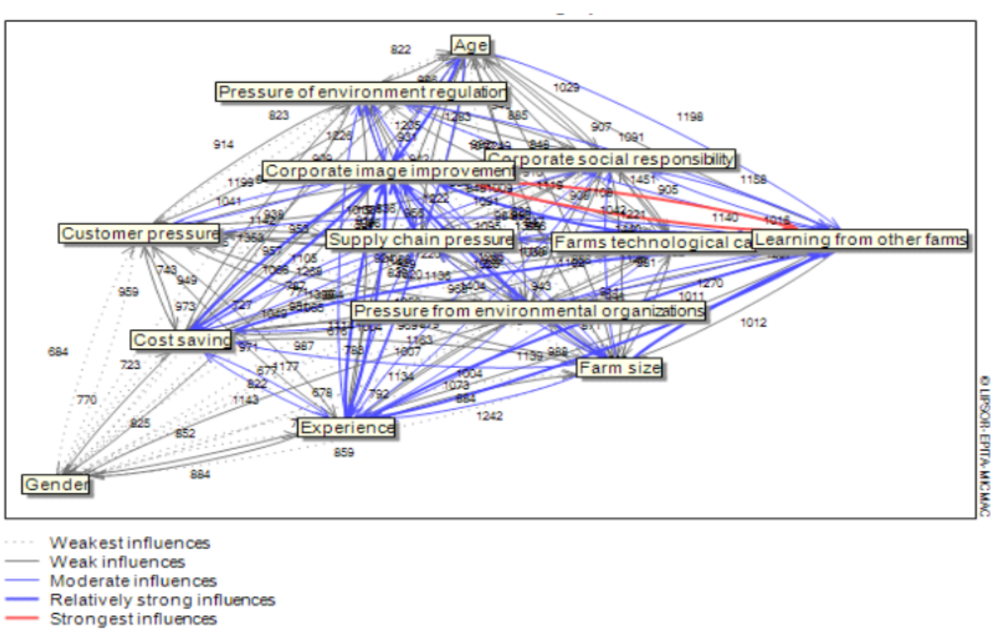

Figure 3. Aggregated cognitive map from indirect influences of the variables influencing pro-environmental behavior.

From the direct and indirect influences-dependences chart (Figure 4), we find that the first quadrant contains influencing variables that directly explain the studied phenomenon, with a large degree of influence and a weak degree of dependence. Farm size emerges as one of the most critical determinants of adopting pro-environmental behavior. Our results are consistent with Vanpoucke *et al*. (2014), as the size of the farm is likely to affect the decision to adopt environmentally friendly and sustainable policies, especially since the theory of legitimacy predicts that the larger the farm, the more obsessed with creating and maintaining a good reputation for it.

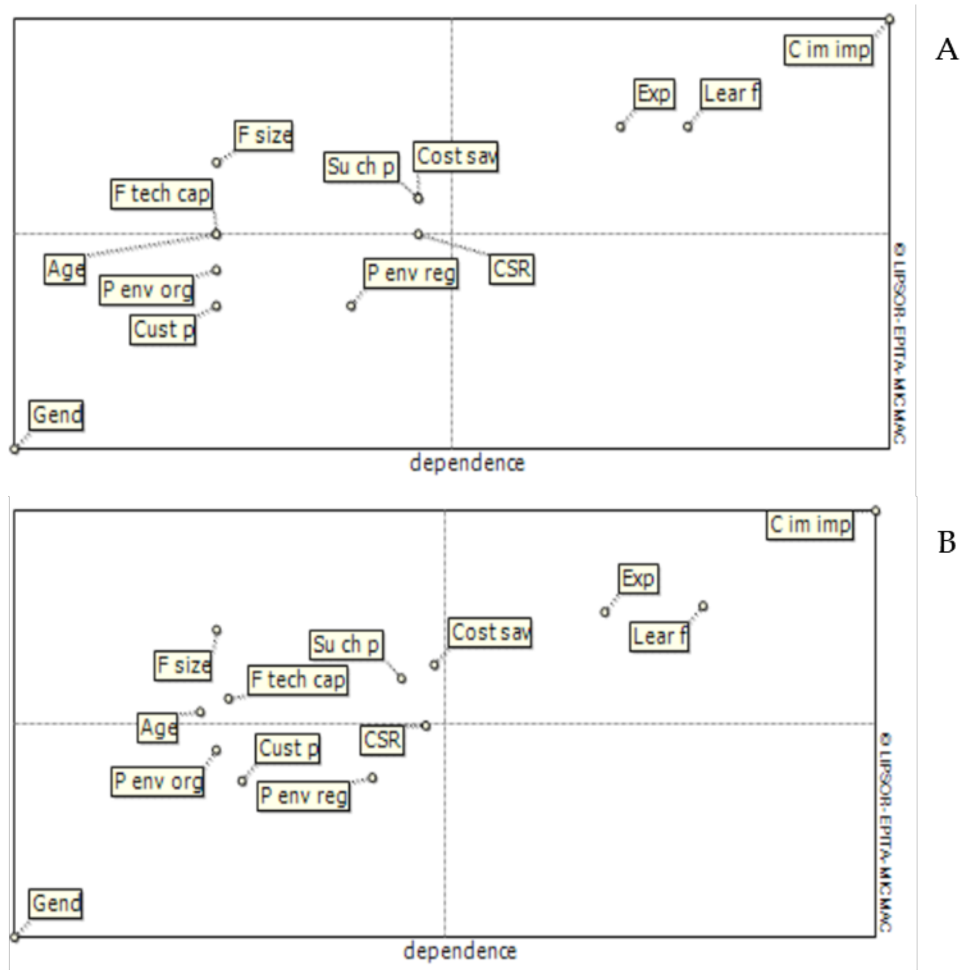

Figure 4. Influences-dependences charts of the variables influencing pro-environmental behavior. A: direct influences-dependences; B: Influences-dependences. F size: farm size; F tech cap: farm technological capabilities; Su ch p: supply chain pressure; Cost sav: cost saving; Exp: farmer experience; Lear f: learning from other firms; C im imp: farm image improvement; Age: farmer age; P en org: pressure from environmental organizations; ust p: customer pressure; P env reg: pressure of environmental regulations; CSR: corporate social responsibility.

Supply chain pressure also has a great impact on the adoption of pro-environmental behavior. In fact, the pressure that supply chains can place on farmers regarding sustainability is considered one of the most important because farmers are simply afraid of losing their position with a particular supply chain if they do not respond to its conditions in terms of sustainability. The pressures of supply chains have a more significant impact on farmers than laws and environmental associations. Our results are consistent with the findings of Bagheri *et al*. (2021), as the principle of cost reduction is a crucial factor in motivating date farmers to adopt pro-environmental behavior.

The technological capacity of the farm and its ability to rely on modern technologies can also explain farmers' adoption of environmentally friendly production policies. This factor is logical, as farms that cannot keep pace with technological developments may not consider adopting pro-environmental behavior, which has been shown to have a strong relationship with technology. These two variables are located on the dividing line between the factors most able to explain the studied phenomenon and the variables that must be excluded. Therefore, we relied on the indirect influences-dependences chart, which suggested that these variables have high explanatory power for the phenomenon of pro-environmental behavior adoption.

The critical variables for adopting pro-environmental behavior are the farm's willingness to create and maintain a good image with various stakeholders, the farmer's experience, and the ability to learn from other farms in the area of sustainability and environmentally friendly practices. These variables can be used to control the system as a whole (Chatziioannou *et al*., 2023). Our results are consistent with Tey *et al*. (2014), who stated that mimetic pressures, represented here in learning from others and emulating them in the field of sustainability, significantly affect their motivation to adopt pro-environmental behavior.

Overall, these results prove the extent to which the three theories discussed can be relied upon in understanding, analyzing, and predicting the phenomenon of pro-environmental behavior. Finally, it was proven that several variables have no direct or indirect relationship with farmers' adoption of pro-environmental behavior in the study sample. These variables are pressure from customers, pressure from environmental protection associations, pressure from environmental protection laws, and gender.

Relying solely on laws to protect the environment and encourage sustainability cannot achieve sustainability in agriculture. On the contrary, more attention should be paid to educating farmers about the importance of pro-environmental behavior and how it can improve their image in front of the relevant parties. It is also necessary to organize periodic forums to support mimetic pressure and push farmers to learn from each other. Awareness of the need to follow social responsibility is also essential and would push farmers to adopt sustainable behavior in the agriculture sector. On the other hand, farmers should be supported technologically and in developing their expertise in the field of dates. Moreover, spreading the culture of green supply chains can significantly help pressure farmers to adopt sustainable policies and behaviors in the field.

Policymakers and extension agencies may create more effective policies and programs to support sustainable agriculture by understanding the variables that drive farmers to embrace these practices. For example, they could offer financial incentives, instruction, and technical assistance to farmers who want to embrace environmentally friendly techniques. Understanding the factors that drive pro-environmental behavior can contribute to the long-term sustainability of date farming in Saudi Arabia. This can help mitigate environmental degradation, preserve natural resources, and ensure the resilience of the agricultural sector in the face of climate change.

CONCLUSIONS

This study attempts to understand the determinants influencing date farmers' decisions to adopt pro-environmental behavior. Our results show that the most critical factors in the date sector are farm size, extent of its technological capabilities, pressure exerted on it by the supply chains in terms of sustainability, obsession with reducing costs, age of the farmer, and the extent of his understanding and desire to follow and apply corporate social responsibility.

The key variables that push farmers to adopt sustainable and environmentally friendly policies and behaviors are the company's desire to create a good image with the relevant parties, the farmer's experience, and the extent to which he can learn from other farms in the field of sustainability. Contrary to what was postulated, we did not find an effect of variables such as pressure on farmers from laws, environmental protection organizations, or pressure from customers. Also, gender was not found to have any effect on the issue of pro-environmental behavior adoption.

Encouraging pro-environmental behavior among farmers through education and peer learning, alongside offering financial incentives and technical support, is essential for sustainable agriculture. Policymakers and extension agencies must understand these drivers to ensure the long-term sustainability of date farming in Saudi Arabia, mitigating environmental degradation and fostering agricultural resilience in the face of climate change.

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Agrociencia

WHAT DRIVES FARMERS TO ADOPT PRO-ENVIRONMENTAL BEHAVIOR? EVIDENCE FROM DATE FARMS IN SAUDI ARABIA

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ABSTRACT

Despite widespread knowledge of agriculture's sustainability challenges, more needs to be known about the factors influencing farmers' adoption of sustainable practices. This study examines the specific drivers of pro-environmental behavior among date farmers. A variable model was developed, and a questionnaire was used to gather farmers' perceptions of the factors influencing pro-environmental behavior on date farms. A hierarchical structure of the identified factors was developed using MICMAC analysis on 81 farmers in the kingdom of Saudi Arabia. Our results show that farm size, supply chain pressure, and firm technological capabilities are the most relevant determinants of pro-environmental behavior on date farms. Also, farmers' experiences, learning from other firms in the sustainability domain, and farm image improvement are critical variables in the system of pro-environmental behavior adoption. Finally, we argue that pressure from environmental regulation and the farmers' age are excluded variables. It is crucial to consider that relying solely on encouragement and laws to protect the environment cannot achieve sustainability in agriculture. More attention should be devoted to educating farmers about the importance of pro-environmental behavior and how it can improve their image in front of stakeholders. It is also necessary to organize periodic forums to support mimetic pressure and push farmers to learn from each other. Because farmers' environments in developing countries tend to be similar, it is possible to generalize these results to some extent. This model unveils a fresh perspective on promoting sustainability in agriculture by using novel analytical techniques to map relationships between pro-environmental actions and their influencing factors.

Keywords: new institutional theory, legitimacy theory, human capital theory, cognitive mapping.

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INTRODUCTION

In recent years, attention has been clearly directed to environmental preservation and sustainability due to climate change, high levels of pollution, and the emergence of many epidemics and pests (Halaburda and Bernaciak, 2020). In particular, pro-environmental behavior, which represents a critical factor in reaching sustainability in various economic activities, has developed as a new term, especially in agriculture. Although there are several studies on the motives that determine the pro-environmental behavior of farmers, there is no explicit agreement about them. As a result, more research is needed, particularly in agricultural areas where farmer behavior and commitment to environmental conservation are directly affected.

This study seeks to determine the factors influencing date farmers' adoption of pro-environmental behavior in the Qassim region in Saudi Arabia. This region is considered the largest producer of dates in the Kingdom and one of the largest suppliers of several types of dates to the global market. The research problem of this study is considered extremely important, especially in light of the Kingdom of Saudi Arabia's Vision 2030, which aims to create a robust agricultural sector that achieves the anticipated sustainability goals and includes agriculture as one of its most crucial pillars. To protect the environment, it is essential to assess how well farmers adhere to the fundamentals of a sustainable lifestyle (Ataei *et al*., 2018). Agriculture is a significant source of income in developing nations. As a result, environmental changes have significant social and economic repercussions (Yaghoubi Farani *et al*., 2021). In order to develop a clean production policy and maintain pro-environmental behavior, this study seeks to identify the most significant factors influencing farmers' behavior on date farms. Dates are one of the nation's key products since palm trees can survive in arid climates, and the Kingdom of Saudi Arabia is one of the greatest palm tree growers.

It is crucial to use environmentally friendly practices in order to achieve sustainability. Long-term management techniques and the adoption of modern agricultural practices are required to guarantee the survival of palm trees under current conditions and future climate scenarios (Elhendy and Al Katani, 2013). The sustainability of date agriculture is dependent on farmers implementing sustainable agricultural practices. Their decision-making is influenced by beliefs, conventions, experiences, values, tribal patterns, educational attainment, and age (Mobeen *et al*., 2016). Therefore, we identified the factors that drive date farmers to adopt pro-environmental behavior and make sustainable decisions. For this, a theoretical framework based on three theories that are most relevant to the issue of pro-environmental behavior was used. Specifically, we relied on the new institutional, legitimacy, and human capital theories.

The new institutional theory suggests that the behavior of actors within an organization or system is influenced by the institutional environment in which they operate. This means that farmers' behavior is not only influenced by their own individual preferences and beliefs but also by the broader institutional context in which they operate, including social norms, values, and regulations. Legitimacy theory suggests that organizations, including farms, must maintain legitimacy in the eyes of stakeholders in order to survive and thrive. Farmers must engage in socially responsible and environmentally sustainable practices in order to maintain legitimacy with consumers, regulators, and other stakeholders. Human capital theory suggests that investments in education, training, and experience can enhance an individual's productivity and contribute to their success. This can be applied to farmers who invest in sustainable agriculture practices, which can increase their productivity and profitability over the long term. These theories help us understand how farmers make decisions on sustainable agriculture practices and how they can be encouraged to adopt more pro-environmental behaviors over the long term.

Previous studies indicate that there are 13 factors that are likely to influence their behavior, derived from the use of the three theories previously discussed. Based on a review of studies that fall under the new institutional theory, five hypotheses were developed. First, customer pressures can affect the farmers' pro-environmental behavior. Tey *et al*. (2014) mentioned that certain customer requirements have to be satisfied in agriculture. Second, the pressure of government regulations has a positive effect on farmers' pro-environmental behaviors. Regulation-related restrictions all have a considerably positive effect on farmers' decisions for organic fertilizer and pro-environmental behavior adoption (Lv *et al*., 2023). According to Li *et al*. (2022), environmental regulations and risk perception both significantly affect farmers' desire to adopt sustainable practices. These pressures are related to competitive relations between farmers and agriculture organizations.

Third, we assume that pressure from environmental organizations has a positive effect on farmers' pro-behaviors. Fu *et al*. (2017) found that group pressure significantly influences the development of individual environmental behaviors. Other studies have affirmed the existence of a positive relationship between pro-environmental behavior and group pressure, such as Banwo and Du (2019). Our fourth hypothesis assumes that learning from others pressure has a positive effect on farmers' behaviors toward agriculture businesses. Thus, it is considered an important factor and helps them follow clean production policies. According to Juárez-Luis *et al*. (2018), pressures have the biggest influence on green practices. Learning from other experiences is primordial in this case (Atanasovska *et al*., 2022). Tey *et al*. (2014) affirm that learning from other firms could influence their behaviors, especially when they know each other and share a common language.

Our fifth hypothesis stipulates that supply chain pressure positively influences farmers to adopt pro-environmental behaviors. Organizations and farms should encourage their employees to become environmentally aware by engaging them in pro-environmental behaviors. Paillé and Boiral (2013) stated that there is growing attention among researchers to find the backgrounds of employees' pro-environmental behaviors at work. In order to satisfy the needs of customers, regulators, and other stakeholders, farmers may be encouraged to adopt more sustainable practices throughout their supply chains.

This study also relied on the legitimacy theory, which implies that the farmer considers polishing his reputation and improving his image in relation to the categories of stakeholders. This is what drives them to adopt pro-environmental behavior in all their decisions and activities. In this regard, Vilkė *et al*. (2021) found that farmers' awareness of environmental responsibility in terms of eco-efficiency and clean production has relevant implications. Farmers may experience negative social or economic effects if they are known in their community for indulging in unsustainable or environmentally harmful practices. They may be discouraged from engaging in unsustainable activities as a result. Farmers are more prone to group pressure, desire to have a good reputation in the group, and anticipate having neighbors' trust (Castro-Campos, 2022). Generally, reputation and image are strong inducers of pro-environmental behavior and can set up a positive feedback loop in which the adoption of sustainable techniques is reinforced by the enhancement of the farmer's reputation.

The effect of farm size on sustainable supply chain management and performance has received little research. Because larger businesses have more resources at their disposal and are subject to more environmental pressure than smaller businesses, company size may have an impact on how well environmental practices are implemented (Wang *et al*., 2023). According to Ren *et al*. (2019), farm size is linked to a statistically significant drop in the amount of fertilizer and pesticides used per hectare. The use of herbicides and pesticides would generally decline by 1.8 % and the use of fertilizers by 0.3 % for every 1 % increase in farm size.

Using previous studies, we developed three hypotheses using this theory as potential determinants of farmers' pro-environmental behavior. The first one stipulates that the engagement of farmers in corporate social responsibility has a positive impact on farmers' pro-environmental behaviors. The second predicts that corporate farm image improvement will positively influence farmers to adopt pro-environmental behaviors. Finally, the third hypothesis related to legitimacy theory predicts that farm size influences farmers positively to adopt pro-environmental behaviors.

On the other hand, human capital theory suggests that there are significant factors that can influence farmers' behavior and their willingness to adopt pro-environmental behavior. Based on this theory, we hypothesized that the farmer's age, time of experience in their field of activity, and gender can largely affect their behavior. Fang *et al.* (2018) found that younger people are more concerned about the environment than older adults. Recent review articles by Glazebrook *et al*. (2020) provide a strong foundation regarding the relationship between gender and environmental concerns in agriculture. Farmers with more experience should be more knowledgeable and proficient when assessing and interpreting information. We concur that farmers with experience are more likely to implement agriculturally pro-environmental practices (Chizallet *et al*., 2023).

Finally, the current study assumes that there are two additional variables that could have explanatory power for farmers' adoption of pro-environmental behavior, which are the technological capability of the farm and the desire to reduce costs. In fact, farms will require the first to adopt pro-environmental technologies, as well as the ability to create projects to execute and modify their processes, operations systems, and information systems (Oke, 2023). Regarding cost savings, Savari *et al*. (2021) confirm that the familiarity of farmers with the benefits of pro-environmental behavior adoption can lead to their increased positive attitude towards pro-environmental behavior, which can directly lead to increasing crop yields. Thus, it is important to accelerate the transformation of agricultural production methods, dynamically develop green agriculture, and protect the environment while increasing incomes (Liu *et al*., 2023).

MATERIALS AND METHODS

The MICMAC analysis technique

In order to determine the most influential factors on the pro-environmental behavior of date farmers, we relied on mind maps using the MICMAC software. In fact, this technique aims to discover how date farmers view the issue of pro-environmental behavior and what factors they see as actually influencing this responsible behavior. If we identify the factors that are likely to influence farmers' behavior and make them adopt sustainable behavior, it is actually easier to direct their behavior in this area.

The structural analysis method was used, specifically the MICMAC method. First, the variables likely to affect the studied phenomenon are identified. Secondly, the adjacency matrix is filled. Third, an adjacency matrix must be entered into the MICMAC software, and the analysis must be performed via the matrix of direct influence and the matrix of indirect influence (Arcade *et al*., 1999). After constructing an individual cognitive map for each farmer, we combined these individual cognitive maps to obtain the aggregated cognitive map, which summarizes farmers' perceptions around the critical determinants of pro-environmental behavior. We applied the arithmetic mean to go from individual to aggregated cognitive maps (Lajnef *et al*., 2017).

A battery of analysis tools was applied to analyze farmers' perceptions. The centrality analysis was used to identify the most critical factors that can influence the pro-environmental behavior. The MICMAC software can easily generate a variable ranking based on the capacity of each variable or concept to influence other concepts in the whole system and its dependence degree in the studied phenomenon. We focused on the centrality analysis, which supposes that in a given system, the most central concept has the highest number of entry and exit arcs. For this, we concentrated on the aggregated cognitive map generated. We used the influences-dependences chart since it can give a clear idea of how all proposed variables as potential determinants of farmers' pro-environmental behavior are clustered in farmers' mental schemes.

The influences-dependences chart (Figure 1) generated by the MICMAC software comprises four quadrants. The first represents the input/influential variables that seem to significantly impact farmers' pro-environmental behavior adoption (Chatziioannou

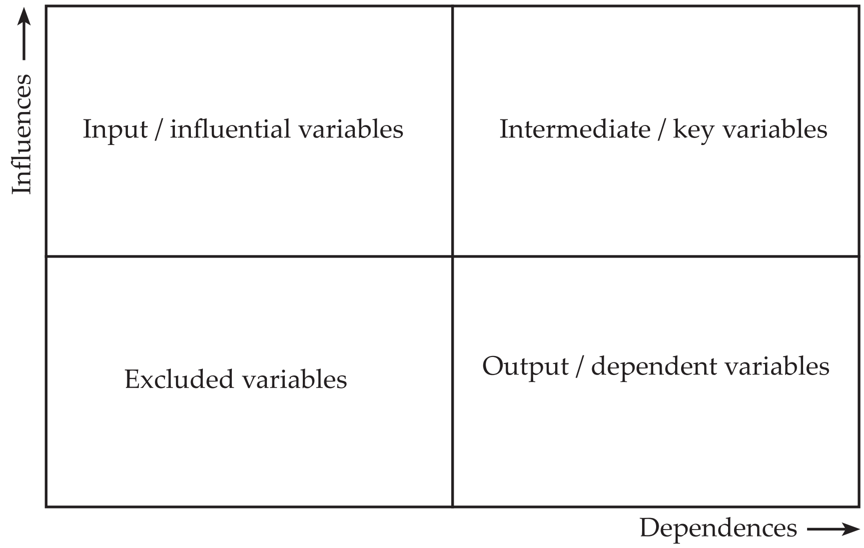

Figure 1. Influences-dependences chart showing the general explanatory value arrangement of the variables.

et al., 2023). Furthermore, these variables are the most influential and can have an explanatory power for the studied phenomenon. The second quadrant contains the relay variables, also called intermediate/key variables due to their ability to influence the whole system (Ben Fatma *et al*., 2021). The third quadrant shows the dependent variables with low influence and strong dependence which have low explanatory power. Finally, the fourth quadrant contains excluded variables that represent variables with no explanatory power, and so are rejected by the system (Chatziioannou *et al*., 2023).

Study area description

This research was carried out in the Qassim region of the Kingdom of Saudi Arabia. It is located approximately 400 km northwest of Riyadh. Al-Qassim is known as the alimental basket of the country due to its distinction in agriculture, especially the production of different types of dates. Data was collected in approximately four months, from January to April 2023. According to official statistics, the Qassim region produces 200 thousand Mg of dates annually (Ben Mohamed *et al*., 2024). This makes the subject of the study particularly important, given the findings of some published studies by Abdallah *et al*. (2018), where the presence of pesticide residues was proven in 18 % of the sample they examined.

Sample collection

The self-structural interaction matrix was produced based on previous studies in the field of pro-environmental behavior. A set of questions was developed to collect basic data about the farmers who participated in the survey, such as age and experience. Before distribution, the questionnaire was presented to a small group of three farmers to ensure its clarity. Then, it was sent to the Ethics Subcommittee and approved. The purpose of the questionnaire was to make it easier to gather information in order to create the SSIM matrix.

The questionnaire was given to a total of 123 date farmers selected at random. At the end, 81 completed replies were obtained. The farmers interviewed were invited to estimate, for each variable and following our theoretical study and exploration of the field, the influence effects by filling out a structural analysis matrix. This matrix contained all potential determinants of pro-environmental behaviors, and farmers were asked to assign the impact of each variable on other variables. If the relationship is strong between two variables, then a value of 3 is assigned. If this relationship is medium, a value of 2 is assigned. If this relationship is weak, its value is 1. If there is no impact between variables, its value is 0.

RESULTS

The sample was heterogeneous in terms of the gender. It was composed of 87.6 % men and 12.4 % women (Table 1). All age groups were present. This is important to study the phenomenon considering all age groups' perceptions. Our sample contained farmers with different education levels and experiences. The questionnaire was distributed to farmers and was designed in a way that enables us to collect general information about the owner of each farm.

By looking at the aggregated adjacency matrix, we can extract the most critical variables central to pro-environmental behavior (Table 2) using the total influence score. The

Table 1. Sample descriptive statistics (n = 81) showing results from the respondents' general information.

Variables	Class	Frequency	Percentage
Age	≤ 30	11	0.135
	31–40	19	0.235
	41–50	18	0.222
	51–60	25	0.308
	> 60	8	0.098
Gender	Male	71	0.876
	Fiemale	10	0.124
Education level	No formal education	17	0.209
	Primary	20	0.247
	Secondary	18	0.222
	Tertiary	26	0.321
Years of experience	≤ 5	24	0.296
	5–10	16	0.197
	11–15	18	0.222
	> 15	23	0.284

Table 2. Adjacency matrix with influence scores assigned to all variables according to their potential impact on each other.

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	Total influences	Total influences and dependences
V1	0	2	1	2	1	1	2	1	2	3	2	2	2	21	44
V2	2	0	2	1	1	1	2	3	2	3	2	3	2	24	48
V3	2	2	0	1	1	1	2	1	2	3	2	2	2	21	42
V4	2	2	1	0	2	1	2	2	2	3	2	3	1	23	44
V5	1	1	1	2	0	2	3	2	2	2	2	3	2	23	44
V6	1	1	1	1	2	0	2	1	2	2	2	1	1	17	35
V7	2	2	2	3	2	2	0	2	2	2	2	3	2	26	53
V8	2	3	2	2	2	1	3	0	1	2	2	2	2	24	45
V9	2	2	2	2	2	2	2	1	0	2	2	2	2	23	47
V10	3	3	3	2	2	2	2	2	3	0	2	3	2	29	59
V11	2	2	2	2	2	2	2	2	2	3	0	2	1	24	48
V12	2	2	2	2	2	2	3	2	2	3	2	0	2	26	54
V13	2	2	2	1	2	1	2	2	2	2	2	2	0	22	43
Total dependences	23	24	21	21	21	18	27	21	24	30	24	28	21		

V_1: pressure of environment regulation; V_2: cost savings; V_3: customer pressure; V_4: farms technological capability; V_5: age; V_6: gender; V_7: experience; V_8: farm size; V_9: corporate social responsibility; V_{10}: corporate image improvement; V_{11}: supply chain pressure; V_{12}: learning from other farms; V_{13}: pressure from environmental organizations.

importance of each variable in the system can be noticed as it measures the extent of the variable's susceptibility to other variables in the system.

Depending on the extent of the variable's effect on the phenomenon of pro-environmental behavior, we find three central variables: corporate image improvement, farmer experience, and the ability to learn from other farms. Especially corporate image improvement, as predicted by the legitimacy theory, can be considered a central variable in the determinants of pro-environmental behavior. This variable has a total score of influences equal to 29, meaning it significantly influences other variables in this system. On the other hand, other studies consider gender to be an essential factor, which may explain why environmentally friendly production policies and behaviors are adopted by certain people. It appears that gender is the weakest variable in terms of its ability to influence the rest of the other variables in the system, according to what farmers see in the study sample, with a score equal to 17.

If we rely on the total influence score to arrange the variables according to their importance, we find that the results are almost constant, especially for the three central variables. Specifically, corporate image improvement is the most affected variable as it receives influences from various other variables in the system, with a total value of 30, followed by the variable of learning from other farms with a score equal to

28. Then, the farmer's experience is greatly affected by the rest of the variables in the system, with a score equal to 27. We also note that three variables are classified as fourth: corporate social responsibility, supply chain pressure, and cost savings. Finally, when taking into account the extent to which a variable can influence and be affected by the rest of the variables, we note that the central variables are: corporate image improvement, learning from other farms, experience, cost savings, supply chain pressure, and corporate social responsibility.

From the aggregated cognitive map (Figure 2), it is possible to notice several influence relationships among the variables that have been proposed to explain the motivation to adopt pro-environmental behavior. The map is overlapping, and several influence relationships exist between variables. These explain that the studied phenomenon has an actual impact on guiding the behavior of farmers in the study sample.

The map shows that the desire to maintain the good reputation, as predicted by the theory of legitimacy, is considered a significant variable, as it affects the majority of other variables in the map and is also affected by them. This indicates the importance of the interpretation proposed by the theory of legitimacy that farmers, in their attempt to build and maintain a good reputation, will adopt pro-environmental behavior to achieve this goal. In this regard, corporate image improvement directs the company towards pressure on costs and the extent of reliance on advanced and environmentally friendly technologies. It also has a considerable impact on the decision to adopt social responsibility and learn from other farms in the field of sustainability.

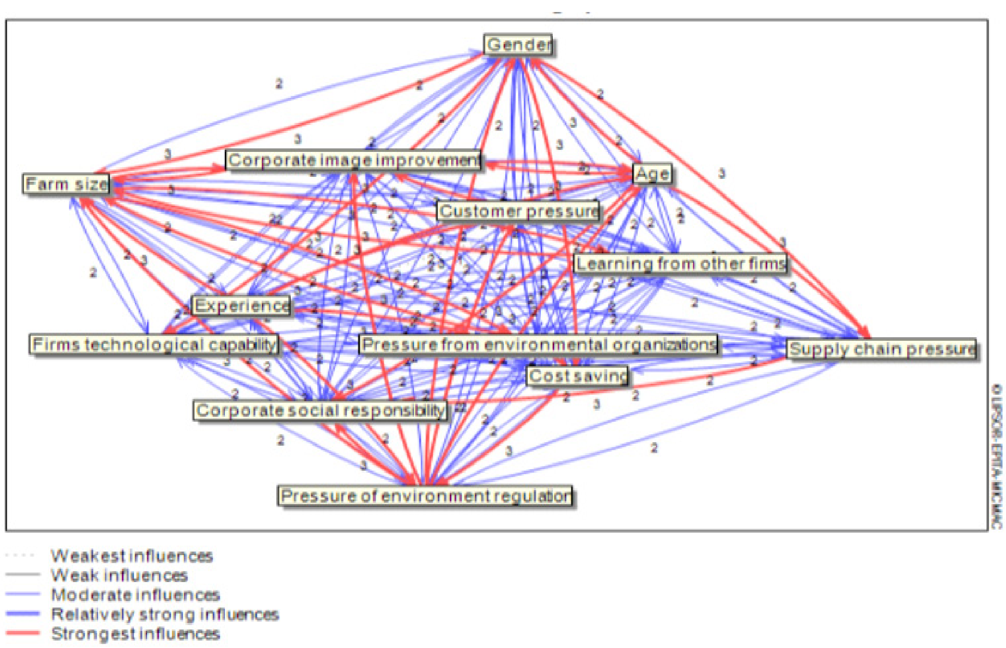

Figure 2. Aggregated cognitive map from direct influences of the variables influencing pro-environmental behavior.

One of the analysis tools that the MICMAC program can provide is the study of indirect relationships. The relationship between the variables goes beyond the limits of direct influence, as there are generally strong relationships between most of the study's variables, except for gender (Figure 3). There is still a robust relationship between creating and maintaining a good reputation for the farm and the ability of the farmer to learn from the experiences of other farms in the field of sustainability. It also appears that the farmer's experience has a strong indirect effect on the majority of the variables in the system, making it a significant variable in the farmers' mental map. Finally, it appears that gender does not significantly affect the rest of the variables that may affect farmers' decisions to adopt pro-environmental behavior. The effect of the rest of the variables on it is also weak.

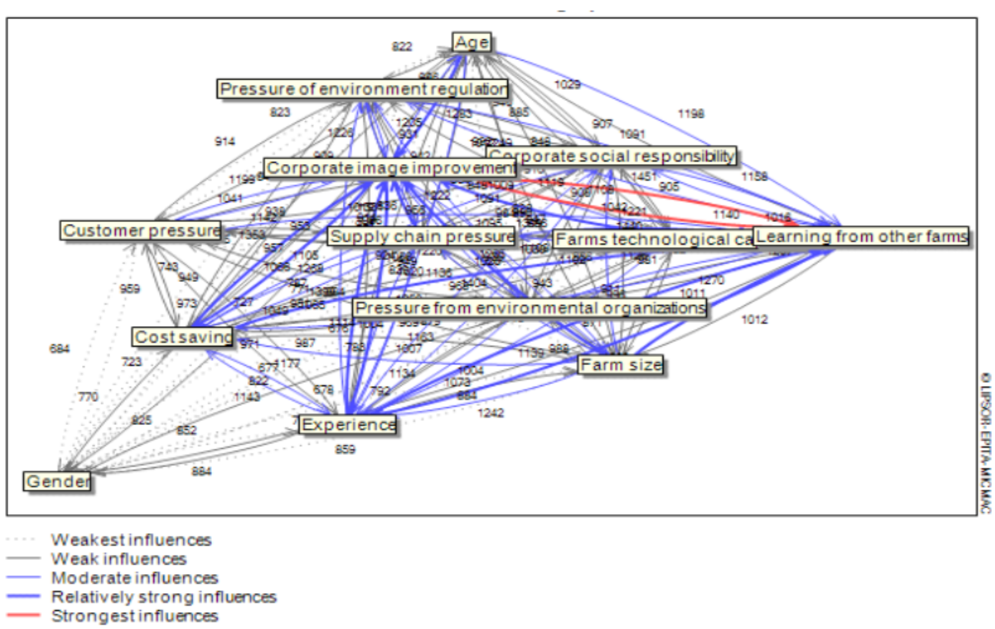

Figure 3. Aggregated cognitive map from indirect influences of the variables influencing pro-environmental behavior.

From the direct and indirect influences-dependences chart (Figure 4), we find that the first quadrant contains influencing variables that directly explain the studied phenomenon, with a large degree of influence and a weak degree of dependence. Farm size emerges as one of the most critical determinants of adopting pro-environmental behavior. Our results are consistent with Vanpoucke *et al*. (2014), as the size of the farm is likely to affect the decision to adopt environmentally friendly and sustainable policies, especially since the theory of legitimacy predicts that the larger the farm, the more obsessed with creating and maintaining a good reputation for it.

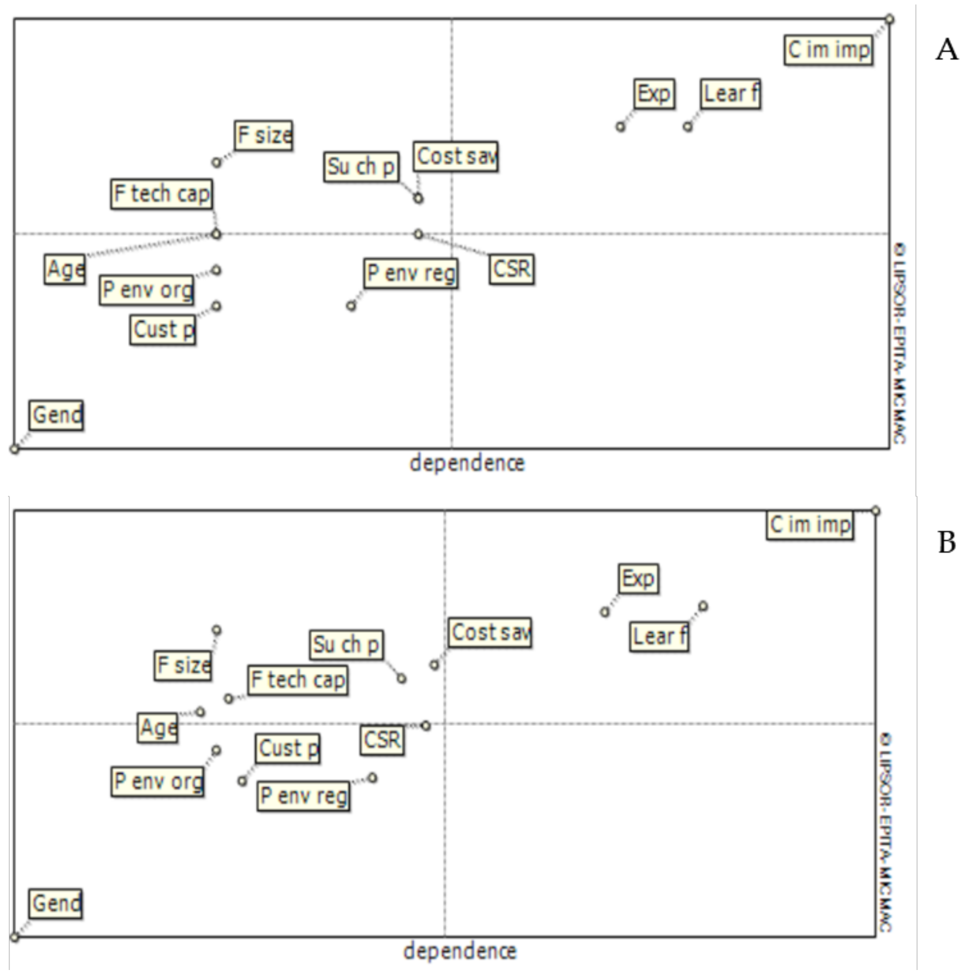

Figure 4. Influences-dependences charts of the variables influencing pro-environmental behavior. A: direct influences-dependences; B: Influences-dependences. F size: farm size; F tech cap: farm technological capabilities; Su ch p: supply chain pressure; Cost sav: cost saving; Exp: farmer experience; Lear f: learning from other firms; C im imp: farm image improvement; Age: farmer age; P en org: pressure from environmental organizations; ust p: customer pressure; P env reg: pressure of environmental regulations; CSR: corporate social responsibility.

Supply chain pressure also has a great impact on the adoption of pro-environmental behavior. In fact, the pressure that supply chains can place on farmers regarding sustainability is considered one of the most important because farmers are simply afraid of losing their position with a particular supply chain if they do not respond to its conditions in terms of sustainability. The pressures of supply chains have a more significant impact on farmers than laws and environmental associations. Our results are consistent with the findings of Bagheri *et al*. (2021), as the principle of cost reduction is a crucial factor in motivating date farmers to adopt pro-environmental behavior.

The technological capacity of the farm and its ability to rely on modern technologies can also explain farmers' adoption of environmentally friendly production policies. This factor is logical, as farms that cannot keep pace with technological developments may not consider adopting pro-environmental behavior, which has been shown to have a strong relationship with technology. These two variables are located on the dividing line between the factors most able to explain the studied phenomenon and the variables that must be excluded. Therefore, we relied on the indirect influences-dependences chart, which suggested that these variables have high explanatory power for the phenomenon of pro-environmental behavior adoption.

The critical variables for adopting pro-environmental behavior are the farm's willingness to create and maintain a good image with various stakeholders, the farmer's experience, and the ability to learn from other farms in the area of sustainability and environmentally friendly practices. These variables can be used to control the system as a whole (Chatziioannou *et al*., 2023). Our results are consistent with Tey *et al*. (2014), who stated that mimetic pressures, represented here in learning from others and emulating them in the field of sustainability, significantly affect their motivation to adopt pro-environmental behavior.

Overall, these results prove the extent to which the three theories discussed can be relied upon in understanding, analyzing, and predicting the phenomenon of pro-environmental behavior. Finally, it was proven that several variables have no direct or indirect relationship with farmers' adoption of pro-environmental behavior in the study sample. These variables are pressure from customers, pressure from environmental protection associations, pressure from environmental protection laws, and gender.

Relying solely on laws to protect the environment and encourage sustainability cannot achieve sustainability in agriculture. On the contrary, more attention should be paid to educating farmers about the importance of pro-environmental behavior and how it can improve their image in front of the relevant parties. It is also necessary to organize periodic forums to support mimetic pressure and push farmers to learn from each other. Awareness of the need to follow social responsibility is also essential and would push farmers to adopt sustainable behavior in the agriculture sector. On the other hand, farmers should be supported technologically and in developing their expertise in the field of dates. Moreover, spreading the culture of green supply chains can significantly help pressure farmers to adopt sustainable policies and behaviors in the field.

Policymakers and extension agencies may create more effective policies and programs to support sustainable agriculture by understanding the variables that drive farmers to embrace these practices. For example, they could offer financial incentives, instruction, and technical assistance to farmers who want to embrace environmentally friendly techniques. Understanding the factors that drive pro-environmental behavior can contribute to the long-term sustainability of date farming in Saudi Arabia. This can help mitigate environmental degradation, preserve natural resources, and ensure the resilience of the agricultural sector in the face of climate change.

CONCLUSIONS

This study attempts to understand the determinants influencing date farmers' decisions to adopt pro-environmental behavior. Our results show that the most critical factors in the date sector are farm size, extent of its technological capabilities, pressure exerted on it by the supply chains in terms of sustainability, obsession with reducing costs, age of the farmer, and the extent of his understanding and desire to follow and apply corporate social responsibility.

The key variables that push farmers to adopt sustainable and environmentally friendly policies and behaviors are the company's desire to create a good image with the relevant parties, the farmer's experience, and the extent to which he can learn from other farms in the field of sustainability. Contrary to what was postulated, we did not find an effect of variables such as pressure on farmers from laws, environmental protection organizations, or pressure from customers. Also, gender was not found to have any effect on the issue of pro-environmental behavior adoption.

Encouraging pro-environmental behavior among farmers through education and peer learning, alongside offering financial incentives and technical support, is essential for sustainable agriculture. Policymakers and extension agencies must understand these drivers to ensure the long-term sustainability of date farming in Saudi Arabia, mitigating environmental degradation and fostering agricultural resilience in the face of climate change.

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EFFECT OF CHEMICAL INDICATORS AND RESPIRATORY ACTIVITY ON THE RESIDENCE TIME OF VERMICOMPOSTS

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ABSTRACT

Vermicompost is considered an environmental quality for managing agricultural residues since it improves soil structure, provides nutrients, and helps to reduce environmental impact. The objective of this study was to determine the residence time using a kinetic model of carbon mineralization while also evaluating chemical and biological parameters obtained during vermicompost processing. Earthworms (*Eisenia fetida*) were used comparing different doses of residual sludge (LR) at 0, 10, 20, and 40 Mg, keeping constant the dose of domestic waste (RD) and cattle manure (EV) at a ratio of 1:1 (dry basis). An AxB factorial design was used, where A represented the LR dose and B the type of residues (RD and EV); thus, eight treatments with nine replicates were compared. The pH, organic matter (MO), total nitrogen, C/N ratio, respiratory activity, C mineralization, and residence time were determined. The results of the treatments indicate a slightly alkaline trend. MO was different among treatments, with a higher MO percentage in EV and LR with 40 Mg of LR (28.92 ± 10.78 %, $F_{(7,88)} = 2.63$, $p \leq 0.01$). The total N percentage was low, but the treatment containing 40 Mg of LR and RD (1.04 ± 0.62 %, $F_{(7,88)} = 3.87$, $p < 0.01$) stood out. C/N ratios < 20, which indicate stability, were recorded in the treatments with LR and EV. The vermicompost obtained from 40 Mg of LR and EV complied with a minimum residence time (less than 70 days) throughout processing, making it an excellent choice for agricultural use.

Keywords: stabilization, half-life, organic amendment, carbon kinetics.

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INTRODUCTION

The growing interest in the use of soil amendments, such as manures, composts, vermicomposts, and biochar, is due to their significant contribution to improving soil structure and their ability to provide nutrients for sustainable agriculture, aligned with the 2030 Agenda for Sustainable Development of the United Nations, where vermicompost plays a key role in mitigating environmental impact (Abbott *et al*., 2018; Kauser and Khwairakpam, 2022). In addition to the interest in environmentally friendly composts, there is social concern about solid waste generation and decreasing its impact on the environment. An integrated management of this type of waste can be carried out, including its reduction, reuse, recycling, thermal energy recovery, and adequate waste confinement (Dümenci *et al*., 2021; Tan *et al*., 2021; Vorobeva *et al*., 2022).

Amendments in organic agriculture offer valuable alternatives by utilizing a wide range of available organic wastes, such as manures, domestic wastes, and residual sludge. The latter are employed due to their contribution of nutrients and organic matter, either directly or after stabilization (Doan *et al*., 2013; Abbott *et al*., 2018; Rékási *et al*., 2023). One solution to waste is vermicomposting, which consists of a bioxidative process in which the earthworm plays a key role and is considered a clean, sustainable, and effective technology to recycle organic waste (Das *et al*., 2022; Ugak *et al*., 2022; Chen *et al*., 2023).

Vermicompost shortens the stabilization time of organic matter and supplies nutrients such as phosphorus, nitrogen, calcium, and magnesium. It also increases moisture holding capacity, microbial activity, and stable organic matter to the soil, increasing crop yields (Das *et al*., 2021; Mahapatra *et al*., 2022; Chen *et al*., 2023; Gebrehana *et al*., 2023). Vermicompost must meet minimum requirements for its production, whose raw material (diverse waste) is generated by agricultural, forestry, and livestock activities, as long as they are biodegradable, do not affect the quality of the final product, and represent no risk to human health or the environment, according to the Mexican standard (DOF, 2007).

The present study responds to the need to understand the vermicomposting process and its relationship with chemical and biological parameters. This understanding is essential to maximizing the efficiency of vermicompost and its capacity to oxidize agricultural residues effectively, so it must adhere to the maturity and stability of the product.Maturity shows to what extent the composting process is completed, while stability refers to a particular state of organic matter, specific stage, or decomposition of organic matter. It is assessed with parameters such as pH, total nitrogen, organic matter, C/N ratio, electrical conductivity, bulk density, and moisture content, among others. It can also be assessed using biochemical parameters such as enzyme activity, respiration, and mineralization, which are indicators of process dynamics (Nikaeen *et al*., 2015; Mahapatra *et al*., 2022).

This research is useful in learning about several chemical and biological factors, including respiratory activity, which is widely utilized as an indicator of soil microbial activity. The metabolic activity of soil microorganisms is determined by quantifying CO_2, whose emission increases in the presence and activity of earthworms because they promote the decomposition and stabilization of the substrate (Chen *et al*., 2023). Similarly, the support of mathematical models with predictions in the operation is required to help optimize costs, calculate time in process efficiency, and extrapolate its effects in the agricultural field (Dümenci *et al*., 2021). The work by Sharma *et al*. (2021) validates the maturity of a vermicompost by applying a response surface model (RSM) and artificial neural network (ANN) with flower residues and cow dung, considering the C/N ratio and CO_2 evolution rate as maturity parameters. The use of kinetic models of carbon mineralization using zero, first, and second-order differential equations helps to predict the degradation of organic matter and its evolution, as well as the residence time or maturation during the process, relying on the respiratory activity expressed as the rate of CO_2 decomposition (Denes *et al*., 2015; Ugak *et al*., 2022).

The hypothesis of this research is that residence time in vermicompost production, together with the application of a kinetic model of carbon mineralization and the measurement of chemical and biological parameters, play a critical role in the quality and efficiency of vermicompost. The relationship between the factors and the composition of the waste used is presumed to influence the final outcome of the vermicompost. Therefore, the objective is to determine the optimum residence time for vermicompost production using *Eisenia fetida* earthworms and evaluate doses of 0, 10, 20, and 40 Mg ha^{-1} of residual sludge (LR), keeping constant the domestic waste (RD) and cattle manure (EV) in the process. This analysis is based on the measurement of chemical and biological parameters to identify the most effective dose and its agricultural use.

MATERIALS AND METHODS

Experimental site location

The study was carried out in the laboratory of the Faculty of Sciences of the Autonomous University of the State of Mexico (UAEMex), located at km 15.5 of the Toluca-Ixtlahuaca highway, State of Mexico, Mexico (19° 24′ 32″ N, 99° 41′ 20″ W).

Solid waste sampling

Organic wastes collected included cow manure (EV) from the post of the Veterinary Faculty of the UAEMex, domestic waste (RD) from the cafeteria of the University campus "El Cerrillo" UAEMex, and residual sludge (LR) from the filter press of the municipal wastewater treatment plant Toluca Norte, which is management by the company Operadora de Ecosistemas S.A. C.V. The pH values, amount of organic matter (MO), organic carbon, C/N ratio, and concentration of various components were recorded for each sample (Table 1).

Vermicomposting

Plastic tubs or containers measuring 20 x 30 x 20 cm (width x length x height) were used, each perforated at the base for oxygen circulation, and a mesh was used at the top to avoid worm escape. In each tub, the worm (*Eisenia fetida*) with a feeding preference

Table 1. Chemical characteristics of organic wastes used in vermicomposting (M ± DE).

Parameter	Domestic waste (RD)	Bovine manure (EV)	Residual sludge (LR)
pH	6.8 ± 0.3	7.4 ± 0.6	7.1 ± 0.4
MO (%)	18.5 ± 0.2	23.3 ± 0.7	30.4 ± 0.4
Organic C (%)	10.7 ± 0.6	13.5 ± 0.9	17.6 ± 0.7
C/N	28.5 ± 1.2	16.5 ± 1.5	9.5 ± 1.1
N (%)	1.1 ± 0.6	2.4 ± 0.8	4.1 ± 1.2
K (%)	0.9 ± 0.5	1.2 ± 0.7	1.4 ± 0.6
Ca (mg kg^{-1})	11.5 ± 3.7	9.2 ± 2.8	10.4 ± 2.7
Mg (mg kg^{-1})	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
Cu (mg kg^{-1})	130.0 ± 2.9	235.0 ± 6.8	490.0 ± 9.9
Zn (mg kg^{-1})	75.0 ± 1.4	65.0 ± 1.9	115.0 ± 1.5
Cd (mg kg^{-1})	3.0 ± 0.6	2.0 ± 0.3	2.0 ± 0.76
Ni (mg kg^{-1})	25.0 ± 1.7	17.0 ± 1	82.0 ± 1.1
Cr (mg kg^{-1})	50.0 ± 0.6	47.0 ± 0.3	124.0 ± 0.6
Pb (mg kg^{-1})	54.0 ± 0.8	31.0 ± 0.1	99.0 ± 0.5

M: mean; SD: standard deviation; MO: organic matter.

of organic matter was added, validating indicators of sexual maturity: individuals of a faint red color, a well-developed clitellum, average size of 4 to 6 cm, and weight of 0.5 g per individual. Twenty adult earthworms per kg of substrate were included, as reported by Gupta and Garg (2008).

To evaluate the stability of the vermicompost, combinations of different types of organic wastes were carried out using an AxB factorial experiment. This study was developed under a completely randomized design that included eight treatments and nine replicates for each of them. The design included the following treatments: T1 = cattle manure (EV) + earthworms; T2 = EV + residual sludge (LR) 10 Mg + earthworms; T3 = EV + LR 20 Mg + earthworms; T4 = EV + LR 40 Mg + earthworms; T5 = domestic residue (RD) + earthworms; T6 = RD + LR 10 Mg + earthworms; T7 = RD + LR 20 Mg + earthworms; T8 = RD + LR 40 Mg + earthworms. The variables considered were the type of waste (animal and domestic) and residual sludge doses of 0, 10, 20, and 40 Mg. The above-mentioned amounts are defined as values that do not exceed the residual sludge standard.

Substrate sampling was carried out for each treatment every 15 d, up to 90 d. Substrate samples were carefully stored in plastic bags for subsequent laboratory analysis. Two reloads of 3.5 kg of organic waste were made at days 45 and 75 to guarantee the necessary food supply for the earthworms throughout the experiment.

Variables evaluated

The samples were dried at room temperature, homogenized, ground, and sieved for chemical analysis in the laboratory to evaluate pH, MO, and total N. The C/N ratio was obtained by dividing the concentration of organic C by total nitrogen. Samples for the study of respiration and mineralization were taken at 15 and 75 d. They were then stored in plastic bags at a temperature of 4 °C, ensuring their conservation until the kinetics were carried out.

Chemical and biological analysis of the vermicompost

The following laboratory parameters were determined: pH using a potentiometer (inoLab® pH 7110, Mexico), total nitrogen (NT) using the Kjeldahl method (Bremner and Mulvaney, 1982), organic carbon (CO) (Walkley and Black, 1934), and the C/N ratio, all based on the Mexican standard (DOF, 2007). Respiratory activity was measured using the Kassem and Nannipieri (1995) method.

Mineralization model to determine residence time

The mineralization process was determined, which required the use of respiratory activity data to generate a kinetic model that considers the available carbon, as represented by the first-order differential equation (Equation 1) proposed by Ugak *et al*. (2022).

$$\frac{dC}{dt} = -KC \tag{1}$$

where dC/dt is the mineralization rate, K is the mineralization constant, and C is the residual carbon concentration.

Numerical calculations yield the residence time, or so-called half-life ($t_{1/2}$), which compares the time required for the potentially mineralizable fraction to decrease its concentration by half (Equation 2). The value 0.693 represents the inverse of ln2 and is obtained by calculating the half-life by solving the associated differential equation. This operation was carried out using the carbon mineralization rate data.

$$t_{1/2} = \frac{0.693}{K} \tag{2}$$

Experiment design and statistical analysis

An AxB factorial design was used, with factor "A" representing the three LR doses used (10, 20, and 40 Mg ha^{-1}) and a control, and factor "B" representing the two types of residues (EV and RD), for a total of eight treatments with nine replicates each. The chemical variable data was analyzed using Statgraphics Centurion XV (Statgraphics.net, Madrid, Spain), and means were compared using Tukey's test (p < 0.05). In addition, the respiratory activity and mineralization of mineralized C were graphed.

RESULTS AND DISCUSSION

Chemical results during vermicomposting process

Significant pH variations were recorded between treatments with and without LR addition ($F_{(7,88)}$ = 3.58, $p \leq 0.02$). The treatment containing 40 Mg of LR and EV, as well as the two treatments without LR addition (EV (7.29 ± 0.23) and RD (7.2 ± 0.24) presented a neutral behavior. However, the other treatments that included different doses of LR, EV, and RD showed a slightly alkaline behavior with pH values between 7.39 ± 0.21 and 7.63 ± 0.27 (Table 2).

Based on this factor, it is reasonable to conclude that the treatment with EV and 40 Mg of LR (T4) has a good potential for agriculture. The Mexican standard (DOF, 2007) establishes a pH range between 5.5 and 8.5 that makes it suitable for use in agriculture. Authors such as Sharma and Garg (2017) and Hassan *et al.* (2022) have shown that vermicompost produced from household waste and horse, cow, and buffalo manures has a pH range of 7.2 to 7.3, thus showing beneficial neutrality levels for plant development. The treatments with these pH values distinguish them notably from the others. The dynamics shown by pH in vermicomposting are attributed to the production of CO_2 and the accumulation of organic acids produced by the microorganisms, and are related to changes in the nitrogen balance. These effects are probably influenced by the presence of earthworms in the vermicomposting process (Hait and Tare, 2011)..

Significant differences ($F_{(7,88)}$ = 2.63, $p \leq 0.01$) in organic matter (MO) concentration were observed among the different treatments. The kind of residue played an important

Table 2. Average values and standard deviation of pH, organic matter (MO) concentration, N and C/N ratio of the different treatments used for vermicomposting.

Treatments	pH	MO (%)	N (%)	C/N
T1	7.29 ± 0.23c	20.32 ± 9.21c	0.29 ± 0.09d	22.36 ± 13.58b
T2	7.52 ± 0.30ab	20.03 ± 7.05c	0.37 ± 0.16d	25.33 ± 13.3b
T3	7.53 ± 0.25ab	22.18 ± 9.20b	0.89 ± 0.96b	16.78 ± 5.66c
T4	7.24 ± 0.38c	28.92 ± 10.78a	0.51 ± 0.34c	19.88 ± 8.39c
T5	7.20 ± 0.24c	16.56 ± 6.14d	0.47 ± 0.15c	37.83 ± 10.37ab
T6	7.41 ± 0.25abc	18.70 ± 8.78cd	0.80 ± 0.04b	37.40 ± 18.61b
T7	7.39 ± 0.21bc	19.00 ± 11.42cd	1.04 ± 0.62a	23.87 ± 9.42bc
T8	7.63 ± 0.27a	15.64 ± 5.73d	0.46 ± 0.06cd	48.86 ± 11.63a

Means with different letters per column represent statistical difference (Tukey $p \leq 0.05$). F: Fisher; *p*: probability. AxB: interaction of A (LR dose) and B (type of residue). Treatments contained combinations of cow manure (EV), earthworms (L), residual sludge (LR), and domestic waste (RD). T1: EV and L; T2: EV and 10 Mg LR; T3: 20 Mg LR and L; T4: 40 Mg LR and L; T5: RD and L; T6: RD and 10 Mg LR; T7: RD and 20 Mg LR; T8: RD and 40 Mg LR.

influence in these discrepancies, with LR and EV exhibiting a larger concentration of MO than RD. For 10 Mg of LR, a value of 20.03 ± 7.05 % was recorded (T2), while for 20 Mg of LR, it was 22.18 ± 9.2 % (T3) and for 40 Mg of LR it reached 28.92 ± 10.78 % (T4). In contrast, those without LR and only with EV presented a value of 20.32 ± 9.21 % (T1), and those containing RD showed 16.56 ± 6.14 % (T5). Treatments combining LR and RD, such as 40 Mg LR with 15.64 ± 5.73 % (T8), 10 Mg LR with 18.7 ± 8.78 % (T6), and 20 Mg LR with 19 ± 11.42 % (T7), exhibited different MO concentrations without showing differences (Table 2). The combination of EV and 40 Mg of LR (T4) stood out for its higher MO concentration, in contrast to the MO concentration found in the mixture of RD and 40 Mg of LR.

The average MO concentration at the end of all treatments was 20.17 ± 9.27 %, which agrees with Ghaffari *et al*. (2022) and the Mexican standard (DOF, 2007). This value indicates an acceptable range of MO between 20 and 50 %, confirming that the results obtained comply with the recommended standards. The decreased proportion of MO in the treatment containing only RD is due to substrate degradation facilitated by the earthworm and mineralization activated by microorganisms, which is consistent with Das *et al*. (2022), Chen *et al.* (2023), and Gebrehana *et al*. (2023). Similarly, Wang *et al*. (2021), Mahapatra *et al*. (2022), and Rékási *et al*. (2023) reported that stable organic residues contribute nutrients such as nitrogen, phosphorus, and potassium to soil in both organic and inorganic forms. However, nitrogen in its organic form is converted to greenhouse gases, which may result in its loss.

Significant differences were observed in the total nitrogen percentage among treatments ($F_{(7,88)}$ = 3.87, $p \leq 0.01$), highlighting those constituted of LR and RD, with higher values of 0.47 ± 0.15 (T5), 0.8 ± 0.04 (T6), and 1.04 ± 0.62 % (T7). Treatments with LR and EV ranked second with results of 0.37 ± 0.16 (T2), 0.89 ± 0.96 (T3), and 0.51 ± 0.34 % (T4). Treatments containing only RD (0.46 ± 0.06 %, T8) and EV (0.29 ± 0.09 %, T1) showed significant differences in nitrogen levels, although with low values (Table 2).

These results underline the significant influence of the combination of LR and RD on nitrogen percentages, followed by the synergy between LR and EV. Specifically, the combination of RD and 20 Mg of LR showed the highest nitrogen percentage, while the lowest concentration was recorded in the EV-only treatment. The importance of carefully considering the composition of the materials utilized is emphasized, as this might have a considerable impact on nitrogen availability in the agricultural system.

Although the average value is below the Mexican standard (DOF, 2007), which has a range between 1 and 4 % N, only T7 met it (1.04 ± 0.6 % N), which was made with LR 20 Mg and RD. According to Ahmed and Deka (2022), Cai *et al.* (2022), Das *et al*. (2021, 2022), and Kauser and Khwairakpan (2022), the addition of nitrogenous mucous substances, decomposing tissues, hormones, and enzymes promotes the earthworm's metabolic activity as well as the production of humic and fulvic acids during the vermicomposting process.

The C/N ratio derived from the quotient between the percentages of carbon and nitrogen exhibits significant differences ($F_{(7,88)}$ = 3.58, $p \leq 0.002$) among treatments, influenced by the amount of LR and the type of residue. The highest C/N ratios are those containing LR and RD, recording 48.86 ± 11.6 for treatment T8 and 37.4 ± 18.1 for T6, including the one containing only RD, with 37.8 ± 10.3. On the other hand, the treatments containing LR and EV presented lower values in the C/N ratio. Ratios of 25.3 ± 13.3 were recorded for the treatment with 10 Mg of LR, 16.7 ± 5.6 for that with 20 Mg of LR, and 19.8 ± 8.3 for that with 40 Mg of LR. This group also includes the treatment containing only EV, with a ratio of 22.3 ± 13.3.

These results highlight the significant influence of the amount of LR residues and the type of residue on the C/N ratio, which plays a crucial role in understanding the decomposition dynamics and nutrient availability in the evaluated systems. Importantly, the composite treatment with 40 Mg of LR and EV exhibits the best chemical stability in terms of C/N ratio, which positions it as an adequate and suitable option for use in agricultural practices. These results provide valuable information to optimize residue management strategies and improve soil quality.

The C/N ratio is often used to evaluate compost stability (Chen *et al*., 2023). Authors such as Katakula *et al*. (2021) and Dümenci *et al*. (2021) mention that a C/N ratio less than 20 indicates good quality vermicompost since soluble MO is biodegradable and contains stable high molecular weight compounds. If the C/N ratio is high, N is deficient to produce bacterial protein and the growth of organisms that decompose organic matter is reduced. Also, Das *et al*. (2021) and Gebrehana *et al*. (2023) explain that the quality of nutrients and the earthworm species present in the waste influence the C/N ratio, which is an important factor in favoring the growth rate of earthworms.

Respiration, carbon mineralization and carbon residence time

The respiratory activity in the treatments represented by factors "A" and "B" shows a similar behavior between them. Likewise, the respiratory activity at the initial time (15 d) (Figure 1A) is higher compared to that observed in the treatments for the final time (75 d) (Figure 1B). At first, the respiratory activity values vary from 640.21 to 814.68 mg CO_2 100 g^{-1} soil. On the other hand, at the end, values ranging from 475.98 to 659.01 mg CO_2 100 g^{-1} soil were recorded.

Respiratory activity at the initial time was notably higher in those treatments containing RD and LR, registering a value of 741.7 ± 48 mg CO_2 100 g^{-1} soil. In contrast, treatments with a combination of EV and LR presented lower respiratory activity, with an average of 616.8 ± 8.6 mg CO_2 100 g^{-1} soil. A similar pattern was observed at the final time, where the treatments with RD and LR exhibited higher respiratory activity, reaching a value of 631.4 ± 29 mg CO_2 100 g^{-1} soil. In contrast, the mixture of EV and LR showed lower respiratory activity, with an average of 516.29 ± 70 mg CO_2 100 g^{-1} soil. This variation can be attributed to the presence of a consortium of microorganisms derived from the waste residues and the vermicomposting process, which is in agreement with Suthar (2009).

It is worth mentioning that the respiratory activity was ascending at the beginning of the vermicomposting process, which is due to an accelerated degradation of the

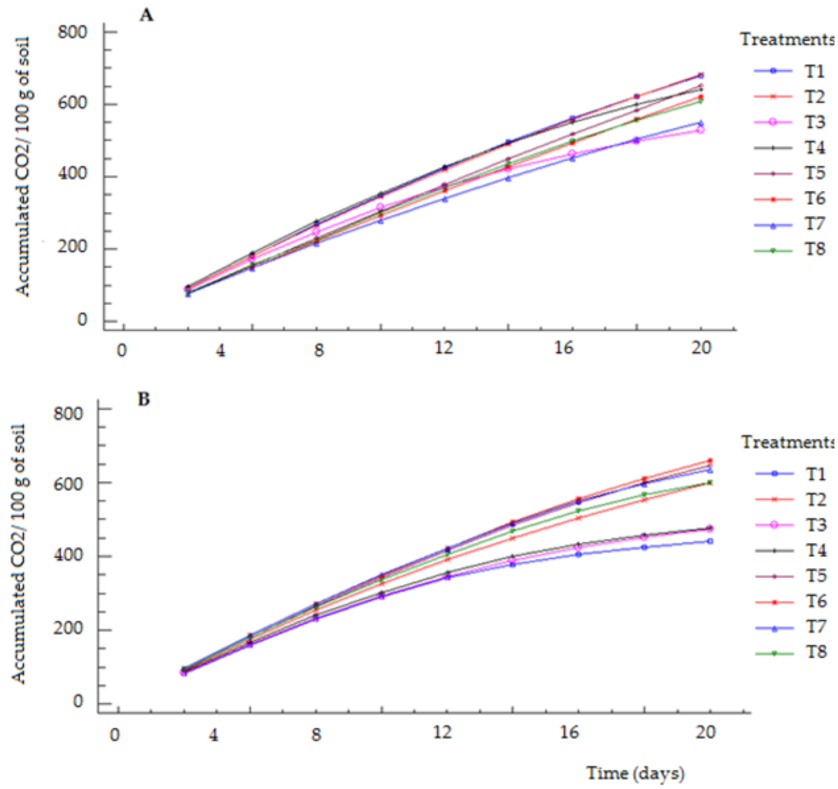

Figure 1. CO_2 evolution of the vermicompost in the eight treatments. A: at 15 d of incubation; B: at 75 d. Treatments contain combinations of cow manure (EV), worm castings (L), residual sludge (LR) and domestic waste (RD): T1 EV and L; T2 EV and 10 Mg of LR; T3 20 Mg of LR and L; T4 40 Mg of LR and L; T5 RD and L; T6 RD and 10 Mg of LR; T7 RD and LR; and T8 RD and 40 Mg of LR.

MO and the continuous feeding of the substrate by the earthworm, coinciding with Gómez-Brandón *et al*. (2022) and Chen *et al*. (2023). On the other hand, the respiratory activity of the treatments reaches a maximum and begins to stabilize, which is due to the decrease in respiratory activity due to the loss of available carbon sources and the presence of compounds rich in lignin and cellulose (Gusain and Suthar, 2020).

Carbon mineralization in the vermicompost

A significant change in carbon mineralization was observed in the treatments containing EV and LR; these mineralized at a faster rate compared to those containing RD and LR from day eight onwards. At this point, mineralization rate showed the following sequence: T4 (K2 = -12.2×10^{-3}) > T3 (K2 = -11.1×10^{-3}) > T1 (K2 = -10.8×10^{-3}) > T2 (K2 = -7.5×10^{-3}). This change possibly suggests a transition from labile to recalcitrant carbon after day eight, which slowed carbon degradation in all treatments.

As of day 10, a significant difference was seen between treatments T5, T6, T7, and T8, which contained RD and LR residues. The mineralization rate sequence in this case was the following: T8 (K2 = -7.59×10^{-3}) > T7 (K2 = -7.37×10^{-3}) > T5 (K2 = -6.81×10^{-3}) > T6 (K2 = -6.57×10^{-3}). This variation is probably related to the amount of organic matter present in each treatment (Figure 2).

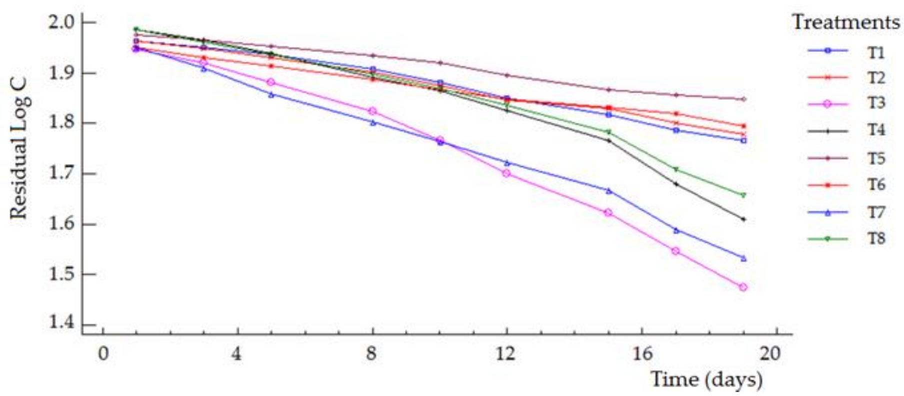

Figure 2. Carbon mineralization rate curves at 75 d of incubation of the treatments. Treatments contained combinations of cow manure (EV), worm castings (L), residual sludge (LR), and domestic waste (RD): T1 EV and L; T2 EV and 10 Mg of LR; T3 20 Mg of LR and L; T4 40 Mg of LR and L; T5 RD and L; T6 RD and 10 Mg of LR; T7 RD and LR; and T8 RD and 40 Mg of LR.

As incubation time progressed, the treatments gradually entered a stage of stability, where the easily degradable material began to be depleted, thus decreasing degradation activity and carbon dioxide release (Alvarez and Alvarez, 2000). In terms of mineralization rate, treatments T1, T3, and T4 demonstrated a higher rate compared to those involving RD and LR, with T4 predominating. According to Chen *et al*. (2023), in order to assess the direct and indirect contributions of earthworms to carbon mineralization, a feeding model incorporating cow dung and stubble residues should be considered and related to the respiration model.

Carbon residence time of the mathematical model

The half-life of the mineralized organic carbon of each treatment was determined, focusing mainly on the K values at 60 d. Based on this information, a classification into three groups was carried out, considering the residence time. The first group covers the interval of $t < 70$ d, where T3 (57 d), T4 (62 d), and T1 (64 d) are included. The second group comprises the interval $70 < t < 100$ d, consisting of T2, T8 (91 d), and T7 (92 d). Finally, the third group encompasses those with $t > 100$ d, being represented by T5 (105 d) and T6 (102 d) (Table 3).

Table 3. Carbon mineralization constants and half-life values at day 75 of the vermicomposting process for each treatment.

Treatments	Mineralization constants K1 and K2		Residence time (d^{-1})
	K_1 (d^{-1}) (10^{-3})	K_2 (d^{-1}) (10^{-3})	
T1	-5.60	-10.80	64
T2	-15.50	-7.58	91
T3	-8.90	-11.10	62
T4	-8.65	-12.20	57
T5	-10.80	-6.81	102
T6	-7.58	-6.57	105
T7	-11.10	-7.37	94
T8	-12.20	-7.59	91

K_1 and K_2: kinetic constants of carbon mineralization. Treatments contained combinations of cow manure (EV), worm castings (L), residue sludge (LR), and domestic residue (RD): T1 EV and L; T2 EV and 10 Mg of LR; T3 20 Mg of LR and L; T4 40 Mg of LR and L; T5 RD and L; T6 RD and 10 Mg of LR; T7 RD and LR; and T8 RD and 40 Mg of LR.

When validating the optimum residence time for vermicompost production, it is observed that the treatments containing EV and LR (T3 and T4) and only EV (T1) allow obtaining vermicompost in less than 70 d. Among these treatments, the one that incorporates 40 Mg of LR and EV (T4) stands out, achieving a time of 57 d and exhibiting ideal chemical and biological qualities for its use in agriculture. Therefore, it is considered that the T4 treatment is not only efficient in producing vermicompost in a reduced time, but also suitable for its application in agricultural soils and environmental improvement.

The model used to determine residence times proves to be practical and useful for predicting the duration of the vermicomposting process, which agrees with Dümenci *et al*. (2021) and Chen *et al*. (2023), who emphasized that mathematical models optimize the process and reduce costs, which benefits agricultural producers.

CONCLUSIONS

Regarding pH, all treatments are slightly alkaline, which suggests that the process is effective at regulating pH. The concentration of organic matter in the treatments is deemed appropriate by the Mexican standard, with a higher percentage in T4 and T3, and the influence of the substances on vermicompost quality is noted. The amount of N in the treatments was low by standard, but its stability was demonstrated by the C/N ratio, which was only met at doses of 20 and 40 Mg of residual sludge and cow dung.

Respiratory activity was more dynamic in the treatments with domestic waste and residual sludge than those containing cow manure and residual sludge. In terms of mineralization rate and residence time, the vermicompost with 40 Mg and cow manure (T4) had an optimal processing time (less than 70 days) and met the standards, making it suitable for agriculture, while the other treatments may have a different purpose, such as forestry or restoration applications.

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