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Croton elegans at Yachay Botanical Garden: population structure and preliminary phytochemistry

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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A mi madre,

mi padre,

mi hermana Joyce,

mi hermano Santiago

mi bello Salem,

mi fea Sabrina,

y a mi amado Cristhian.

Mishu S. G.

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RESUMEN EJECUTIVO

Croton elegans Kunth es una especie endémica común de los valles secos interandinos del Ecuador. Aunque no está amenazada, las propiedades medicinales que tiene esta especie, junto con su rol ecológico, justifican estudios sobre la estructura poblacional y propiedades fitoquímicas de esta especie.

En este estudio, primero realicé un censo de población basado en 32 transectos de vegetación en la Loma de San Eloy (área de conservación) del Jardín Botánico Yachay, en Urcuquí. Para cada planta se registró la altura, cobertura, índice de vecindad interespecífico y estado fenológico. Se censaron un total de 1604 individuos (promedio = 50 plantas por transecto; rango: 9–97). En general, el índice de vecindad fue menor donde la densidad de plantas fue mayor, y las plantas con menor altura fueron más abundantes hacia la cima de la loma, donde los individuos también tenían poca cobertura. Un análisis de componentes principales indicó que las variables morfométricas estaban en su mayoría correlacionadas. Estos resultados sugieren que los factores ambientales correlacionados con el gradiente altitudinal, junto con la competencia interespecífica potencial, pueden afectar la estructura poblacional y las características morfométricas de *C. elegans*.

En segundo lugar, realicé un análisis fitoquímico cualitativo y una evaluación cuantitativa del contenido total de fenoles y contenido total de flavonoides de extractos metanólicos de *C. elegans*, con base en plantas recolectadas bajo las siguientes cuatro condiciones dadas por la distribución natural de esta especie a lo largo de la Loma de San Eloy (datos del censo): (1) plantas alrededor de la cima de la loma con índice de vecindad alto; (2) plantas alrededor de la cima de la loma con un índice de vecindad bajo; (3) plantas alrededor de la base de la loma con índice de vecindad alto; y (4) plantas alrededor de la base de la loma con un índice de vecindad bajo. Para cada una de estas condiciones, se recolectaron hojas de tres plantas representativas (N total = 3 plantas × 4 condiciones = 12 extractos). Todos los extractos metanólicos de las diferentes plantas tenían los mismos metabolitos secundarios, lo que significa que las cuatro condiciones definidas anteriormente no afectaron significativamente la química secundaria de las plantas. Concluyo que el pequeño rango altitudinal de la Loma de San Eloy no afecta considerablemente la expresión cualitativa y cuantitativa de metabolitos secundarios en la población local de *C. elegans*.

Palabras clave: *Croton elegans*, gradiente altitudinal, índice de vecindad, metabolitos secundarios, Ecuador

EXECUTIVE SUMMARY

Croton elegans Kunth is an endemic common species of the inter-Andean dry valleys of Ecuador. Although not threatened, the medicinal properties that this species has, together with its ecological role, justify studies on the population structure and phytochemical properties of this species.

In this study, I first conducted a population census based on 32 vegetation transects at San Eloy Hill (conservation area) at Yachay Botanical Garden, in Urcuquí. For each plant, height, cover, inter-specific neighborhood index and phenology status were recorded. A total of 1604 individuals were censused (average = 50 plants per transect; range: 9–97). In general, neighborhood index was lower where plant density was higher, and shorter plants were more abundant towards the hilltop, where individuals also had low cover. A principal component analysis indicated that morphometric variables were mostly correlated. These results suggest that environmental factors correlated with the altitudinal gradient, along with potential inter-specific competition, may affect the population structure and morphometric characteristics of *C. elegans*.

Secondly, I conducted a qualitative phytochemical screening and a quantitative assessment of total phenolic content and total flavonoid content of *C. elegans* methanolic extracts, based on plants collected under the four following conditions given by the natural distribution of this species along San Eloy Hill (census data): (1) plants around hilltop with high neighborhood index; (2) plants around hilltop with low neighborhood index; (3) plants around hill base with high neighborhood index; and (4) plants around hill base with low neighborhood index. For each of these conditions, leaves of three representative plants were collected (total N = 3 plants x 4 conditions = 12 extracts). All methanolic extracts from the different plants had the same secondary metabolites, meaning that the four conditions above defined did not significantly affect the plants secondary chemistry. I conclude that the small altitudinal range of San Eloy Hill does not considerably affect the qualitative and quantitative expression of secondary metabolites in the local *C. elegans* population.

Keywords: *Croton elegans*, altitudinal gradient, neighborhood index, secondary metabolites, Ecuador

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CHAPTER I

CHAPTER I. Population structure of the endemic species *Croton elegans* Kunth in an inter-Andean dry forest of Urcuquí, Imbabura, Ecuador

ABSTRACT

Croton elegans Kunth, locally known as "mosquera", is an endemic species of the inter-Andean dry valleys of Ecuador with a threat category of "Least Concern". Although it is not seriously threatened, the medicinal properties of this species and its ecological role, justify a deeper scientific understanding of the population structure of *C. elegans* along its distributional range. For this purpose, a census of the population of *C. elegans* was conducted at "Loma San Eloy" (San Eloy Hill), which is part of the *in situ* conservation area of Yachay Botanical Garden, in Urcuquí, Imbabura – Ecuador. Along the slope of the hill, 32 vegetation transects were established: 12 transects at altitudes of 2221 – 2255 masl, and 20 transects at altitudes of 2316 – 2366 masl. For each plant, the following variables were recorded: Height of the main stem, Cover (ellipse area of the canopy), relative inter-specific Neighborhood Index (created ad hoc), and Phenological Status. A total of 1604 individuals of *C. elegans* were recorded in the 32 transects, with an average of 50 plants per transect (range: 9–97). It was observed that in sites with higher density, regardless of altitude, the inter-specific Neighborhood index was low ($\rho = 0.85$, $P < 0.001$), and that relatively short plants were more common towards the hilltop, where individuals also tended to present relatively low cover. A principal component analysis indicated that the morphometric variables are correlated with each other. The results suggest that environmental factors correlated with altitudinal gradient, and potential inter-specific competition, may affect the population structure and morphometric characteristics of *C. elegans*.

Keywords: *Croton elegans*, density, morphometry, phenology, Ecuador

1. INTRODUCTION

The *in situ* conservation of biodiversity aims to maintain the diversity of species, habitats and interrelationships in ecosystems including ecosystem services[1,2]. However, global-scale problems mainly related to the change in land use for agricultural purposes, pastures, or urban sprawl have negative consequences on ecosystems and their biodiversity, but these are not fully understood[3,4]. One alarming and notably significant consequence in many anthropically modified ecosystems is the loss of unique species. Furthermore, the loss of intraspecific genetic diversity in these ecosystems is also worrying because it is not directly measurable and can be difficult to detect in the short-term, thus jeopardizing the long-term viability of populations[5]. In this respect, many genetic resources can be lost, even before their inter- and intraspecific variability is measured and their potential applications (e.g., alternative food, medicine, biofuel, pest control, among others) are elucidated[6].

In Ecuador, one of the most threatened ecosystems is the interandean dry forest/scrub vegetation that covers approximately 59000 km^2 within the valleys of Chota (Imbabura), Guayllabamba (Pichincha), Ambato (Tungurahua), Jubones (Azuay) and southern Loja, among others[7]. The dry interandean valleys are unique in diversity and endemism of plants and animals: in fact, up to a third of the angiosperm species in these valleys can be endemic![8]. However, most dry interandean valleys, indeed biodiversity hotspots[9], now survive only in small patches of vegetation due to thousands of years of agriculture (and nowadays, urbanism) settled in the Andean mountains[10].

In northern Ecuador, one emblematic and endemic plant species from the dry interandean forest is *Croton elegans* Kunth, which is classified in the IUCN conservation category of "Least Concern"[11].The *Croton* genus is among the most diverse in the Euphorbiaceae family, comprising approximately 1300 species[12,13]. *Croton* is also a taxonomically important genus because it has a great life-form diversity that includes herbs, shrubs, trees and lianas[12,14]. It occurs mostly in tropical regions worldwide[12,13,15], although it also has some representatives in subtropical and northern temperate areas. Its main biogeographic hotspots in the Neotropics are Brazil, the West Indies, and Mexico[13]. In Ecuador, according to the Tropicos database[16], there is a total of 38 *Croton* species distributed around the four geographic regions of the country: Amazonia (9 spp., including 1 endemic spp.), Sierra (27 spp., including 9 endemic spp.), Costa (10 spp., including 5 endemic spp.) and Insular (1 endemic sp.)[16]. Note that some of these species

may be widely distributed and may occur in more than one geographic region. From the 38 species reported for Ecuador, from which 15 are endemic, only five species (13%) are considered threatened: four are categorized as "Endangered" and one, "Vulnerable"[11].The main threats that affect the populations of these species include the continuous reduction of habitat caused by anthropogenic fire, introduction of species such as cypress and pine, deforestation by expansion of agriculture and urbanism, and grazing[11].

Ecologically, the species of *Croton* are also important because of their role as pioneers in disturbed forest vegetation in the tropics[17,18]. This ecological characteristic is ideal for restoration efforts, such as the case of *Croton urucurana* Baill in Brazil[18]. In xeric environments, *Croton* is considered a key ecosystem engineer because it can increase local diversity by providing refuge, nectar and food for animals, shelter (nurse effect) and seed trapping for plants[19–21]. In addition, *Croton* species have been used traditionally as natural remedies by indigenous cultures around the world, due to its wide range of pharmacological properties. Popular uses of *Croton* include treatment of many diseases such as cancer, digestive ailments, inflammation, diabetes, hypertension, malaria, external wounds, fever, pain, ulcers, abscesses, among others[12,22].

Even though there is considerable information about the ethnobotanical and biochemical characteristics of several *Croton* species, it seems ironic that the ecology of many of these species is relatively unknown, except for a few studies (*e.g.*, *Croton alabamensis*[23]). Indeed, data on local abundance patterns, population dynamics, phenological cycles and plant-plant interactions, can become extremely useful to design sustainable harvest initiatives from wild (non-cultivated) populations of a species, while protecting its long-term existence and the integrity of the ecological processes.

In this study, the population structure of *C. elegans* at San Eloy Hill, part of the dry forest conservation area located at Yachay Botanical Garden (YBG), Urcuquí, Imbabura, was studied. For each plant, the following traits were measured or assessed: Height, Cover, a "Neighborhood Index" (an estimate of inter-specific competition), and reproductive Phenological Status (see Methods for more information). Based on these data, the following questions were posed (as they apply within the sampling period of this study):

Question 1 (at the transect-level): How does plant density change along the elevation gradient of San Eloy Hill, at the time of sampling?

Question 2 (at the individual-level): What is the statistical distribution of the different structural variables measured or assessed for every plant?

Question 3 (at the individual-level): How do the different population structure variables measured or assessed for every plant relate among each other?

Question 4 (at the individual-level): Is the elevation gradient along San Eloy Hill related, at the individual level, to any of the population structure variables observed in the system?

Question 5 (at the transect-level): Is the elevation gradient along San Eloy Hill related, at the transect level, to any of the population structure variables observed in the system?

Question 6 (at the transect-level): At the transect level, is there any relation between total plant density (N° plants per transect) and the density of plants with different "neighborhood indexes"?

The results of this study will serve as background information for future population dynamics and phytochemical studies aimed to wisely manage the local ecological resources available at YBG´s *in situ* conservation area.

2. MATERIALS AND METHODS

2.1. Study area

The study was conducted between June and November 2021, at San Eloy Hill within the *in situ* conservation area of Yachay Botanical Garden (YBG), in Urcuquí, northern Andes of Ecuador (geographic coordinates at the hilltop: 0°25'03" N 78°11'04" W) (Figure 1). The conservation area at San Eloy Hill ca. 0.6 km^2 and has an altitudinal range from ca. 2200 to 2345 masl. The native ecosystem that naturally occurs at YBG is officially classified as "bosque y arbustal semideciduo de los valles del norte" (semideciduous forest and shrubland of the northern valleys), also known as dry interandean valley vegetation (Ministerio del Ambiente del Ecuador, 2013) [24]. At the hilltop of San Eloy Hill, vegetation tends to be short and dispersed, while at the hill base, vegetation tends to be taller, has higher density and has higher species richness. At the species level, some differences along the elevation gradient can also be detected: for example, the small shrub *Arcytophyllum* cf. *aristatum* (Ruiz & Pav.) Standl. (Rubiaceae)

only occurs towards the hilltop, while the herb *Alternanthera porrigens* Kuntze (Amaranthaceae) tends to occur towards the hill base. These distribution patterns could be explained by historical land use or microclimatic constraints.

Regarding climate, the most trustful data in the area is that from Ibarra [1991-2021][25], the capital province, located at 10 km from the study area in Urcuquí (Figure 2). Mean interannual monthly temperature varies from 15.6 °C (June/July) to 16.7 °C (September). Minimum temperatures tend to occur in August (ca. 11 °C) while highest temperatures tend to occur in September (ca. 23 °C). Average yearly rainfall is ca. 1700 mm/year, with monthly rainfall ranging from 75 mm (August) to 218 mm (April). Historically, the dry period starts around June, intensifying in August/September.

2.2. Plot size and sampling design

To sample the *C. elegans* population at San Eloy Hill, a total of 32 transects (20 m × 2 m) were randomly established and georeferenced within two elevation zones: 2221 – 2255 m (N = 12 transects) and 2316 – 2366 (N = 20 transects) (Figure 3). The number of transects (replicates) per elevation zone is different because it was logistically difficult to increase the number of transects at the lower zone due to high vegetation density. All *C. elegans* individuals, independently of size, were sampled and photographed. For each plant, the following characteristics (variables) were registered: (1) Height (cm) of main stem; (2) Cover (cm^2); (3) Inter-specific Neighborhood Index (created ad hoc for this study); and (4) Phenological Status. Plant Height was defined as the vertical distance from soil level to the tallest apical meristem. Plant Cover was measured as the area of an imaginary ellipse covering the whole plant canopy; thus, to calculate the ellipse area, two measurements were taken: length of longest canopy axis and, at 90° from it, length of the short canopy axis.

The Inter-specific Neighborhood Index was defined as a categorical variable to qualitatively describe how crowded a *C. elegans* plant was, given the spatial closeness of other *different* plant species surrounding it. Possible values where:

- 1: low plant “inter-specific crowdedness” around a plant;
- 2: “typical” plant “inter-specific crowdedness” around a plant; and,
- 3: high plant “inter-specific crowdedness” around a plant.

Phenological Status was defined as a nominal (categorical) variable to indicate the phenological state of a plant at the time of sampling. Possible values where:

- 0: absence of flower buds, open flowers and fruits;
- 1: presence of flower buds only;
- 2: presence of open flowers only;
- 3: presence of fruits only;
- 4: presence of flower buds and open flowers;
- 5: presence of flower buds and fruits;
- 6: presence of open flowers and fruits; and,
- 7: presence of flower buds, open flowers and fruits.

To reconfirm the taxonomic identification of *Croton elegans*, a botanical voucher was collected (M. Sánchez-García 0001) and its taxonomic identification confirmed at the National Herbarium of Ecuador (QCNE) in Quito.

2.3. Data analyses

An Excel® database was used to record and organize the data taken for every plant sampled. Plant density was calculated using pivot tables in Excel and a Spearman correlation was conducted to evaluate if there was any correlation of plant density versus elevation (**Question 1**). To describe the patterns of population structure and reproductive phenology at the individual level (**Question 2**), descriptive statistics of the variables Height (cm) of main stem, Plant Cover (cm^2), Inter-specific Neighborhood Index, and Phenological Status, were conducted. Descriptive statistics (mean, median, mode, range, standard deviation, kurtosis and skewness) were only calculated for the two quantitative and continuous variables: Height and Cover.

In addition, a normality test (Shapiro-Wilk) for each variable was conducted to decide whether parametric or non-parametric tests were more appropriate to analyze the data. Because non-normality was common, Spearman correlation analyses were conducted to detect possible pairwise correlations between all population structure variables (**Question 3**); a significance alpha level = 0.05 was set *a priori* to decide whether a significant correlation existed or not. Very high sample sizes ($N > 1000$) may result in very low P-values ($P < 0.001$), but this apparent correlation may not necessarily represent the underlying true pattern. Therefore, to broadly assess this sampling effect, each Spearman correlation analysis was re-run five times but only with a sample size of $N = 100$ individuals, randomly selected (with replacement).

To assess the possible effect of elevation on the population structure variables (**Question 4**), Spearman correlations were also conducted between elevation and each of the population structure variables measured. These analyses were complemented by a Principal Component Analysis (PCA) that summarized the variation, at the transect level,

of all variables in the system, including elevation (**Question 5**). For the PCA, variables were first centered and standardized (mean = 0; standard deviation, SD = 1), given their differences in scale and magnitude.

Finally, a Spearman correlation between plant density for each transect and number of plants with each Neighborhood Index was conducted (**Question 6**).

All analyses, and graphs associated, were conducted using PAST®, InfoStat® and Minitab Statistical Software®.

3. RESULTS

Note that the figures, tables, and supplementary material of the Results Section are shown at the end of this chapter.

3.1 Question 1: Transect-level statistics of population density

A total of 1604 individuals of *Croton elegans* were registered in the 32 transects established (each 20 m × 2 m: 40 m^2) which represent a sampling area of 1280 m^2 (0.128 ha). The 12 transects at the lowest altitudinal range (2221 – 2255 masl) had 565 individuals, while the 20 transects at the highest altitudinal range (2316 – 2366 masl) had 1039 individuals. Figure 4 shows the density of individuals per transect along the elevation gradient (mean = 50.125 ± 20.5 individuals [± 1 SD]; range = 9 – 97 individuals), while Supplementary Table S1 shows the specific geographic coordinates and plant density for each transect. There was no correlation between plant density and elevation ($\rho = 0.14$, $P = 0.4427$; Figure 4).

3.2 Question 2: Individual-level statistics of population structure variables and reproductive phenology

Mean Height of the *C. elegans* population was 34.3 ± 13.1 cm (± 1 SD; range = 10 – 118 cm). Height distribution was more or less symmetrical because the median (33 cm) and the mode (30 cm) were relatively similar to the mean (Figure 5). This is reflected by a kurtosis value (= 3.5) that is relatively close to that of a normal distribution (= 3). Yet, the distribution does show a positive skew (= 1.4), *i.e.* a tail to the right, because of the relatively low frequency of relatively tall individuals (Figure 5a).

Mean Cover was 3256.6 ± 3178.8 cm^2 (± 1 SD; range = 44 – 23713 cm^2), with a median (2328 cm^2) and a mode (1979 cm^2) relatively different to the mean (Figure 5b).

Note the very large difference between the individual with the lowest cover and that with the highest cover. The J-shaped distribution indicates that the vast majority of individuals have relatively low cover, and that individuals with high cover are very rare. This pattern is also reflected by the high positive skewness (= 2.1) and kurtosis (= 6.5) values (Figure 5b).

Regarding the Inter-specific Neighborhood Index, the most common value was 1 (Figure 5c), indicating that most of the *C. elegans* individuals grew surrounded by relatively few plants from other species. Recall that this index represents an ordinal variable of three possible mutually-exclusive values: 1, 2 or 3.

Finally, regarding the Phenological Status (Figure 5d), it is worth noticing that, during the sampling period (June – November 2021), only 11 plants had a category "0", i.e. they were not fertile at all. The most common category was "7" (N = 565 individuals), which represented 35.2% of the sampled plants. This category indicates the presence of bud flowers, open flowers and fruits, *i.e.* the presence of all reproductive stages in the same plant. The next most common category was "1", with N = 426 individuals (26.6%), followed by categories "4" (N = 358 individuals; 22.3%) and "5" (N = 242 individuals; 15.1%). Recall that category "1" corresponds to plants with only bud flowers present, while categories "4" and "5" also correspond to the presence of flower buds, but with the added presence of either flowers or fruits, respectively.

3.3 Question 3: Spearman correlations among population structure variables

Figure 6 shows all pairwise Spearman correlations among the measured population structure variables measured for each individual plant of the *C. elegans* population studied (N = 1604 individuals). Except for the correlation between Neighborhood Index and Phenological Status (ρ = -0.04, P = 0.1002), the rest of pairwise correlations were highly significant ($P < 0.001$). However, the clearest correlation (high ρ, low P) was between Height and Cover ($\rho = 0.61$, $P < 0.001$)— indeed, an evidently, taller plants had higher cover. The rest of pairwise correlations just presented ρ-values between 0.16 and 0.42, *i.e.*, all were positively correlated, although not too strongly[26].

Note that the patterns above described were detected for a very high sample size (N = 1604 individuals). This huge sample size may however become a statistical bias by "artificially" lowering the P-value, and thus increasing the risk of committing an error on the statistical conclusion. As explained in Methods, we counteracted this possibility by

randomly selecting sets of 100 individuals and re-running the Spearman analyses with them. The results of these analyses show that those correlations that were statistically significant with $N = 1604$ are still significant with $N = 100$, except for particular random cases between Cover and Neighborhood Index, in which some P-values were > 0.05 (Supplementary Table S2).

3.4 Question 4: Spearman correlations between elevation and population structure variables at the individual level

Figure 7 shows all pairwise Spearman correlations between Elevation and each of the population structure variables measured. Except for the correlation between Elevation and Neighborhood Index ($\rho = -0.04$, $P = 0.1530$), the rest of pairwise correlations were highly significant ($P < 0.001$). Although all ρ values were notoriously low, the trend showed that as Elevation increased, Height and Cover of the plants decreased (negative correlation), but the Phenology Index increased (positive correlation). Note that at low altitudes, there were both short and tall plants, but at higher altitudes, only relatively short plants existed (Figure 7). These trends were also maintained in the sub-sampling analyses ($N = 100$ individuals) that were conducted to "control" for the effect of the original very high sample size ($N = 1604$ individuals) (Supplementary Table S3).

3.5 Question 5: Principal Component Analysis between elevation and population structure variables at the transect level

Figure 8 shows the Principal Component Analysis (PCA) that was conducted to understand, at the transect level, the relation of Elevation with the different population structure variables. Principal Component 1 (PC1) represented 46.4% of the variability in the *C. elegans* population. PC1 is mostly related to the variables Elevation (m), Height (cm) and Cover (cm^2). Principal Component 2 (PC2) explained 25.8% of the variability and seemed mostly related to the Phenology Index variable. Thus, PC1 and PC2 explained 72.2% of the total variability in the system, based on the variables chosen in this study to describe the population structure of the *C. elegans* population (Figure 8).

3.6 Question 6: Spearman correlations between density and neighborhood indexes at the transect level

Figure 9 shows all pairwise Spearman correlations between Plant Density and Neighborhood Index at transect level, depicting the behavior that the *C. elegans* population took around the three different values that the Neighborhood Index can take (1, 2 or 3). On the one hand, Figures 9a and 9b, which respectively correspond to the correlations between Plant Density and the No. of plants with values 1 or 2 of the Neighborhood Index, describe highly significant correlations ($\rho > 0.5$, $P < 0.001$). In both cases, a notorious trend showed that as long as the Neighborhood Index remained low or moderated, a major presence of individuals per transect would be evident. On the other hand, Figure 9c, which represents the correlation between Plant Density and the No. of plants with Neighborhood Index with value 3, showed no significant correlation.

4. DISCUSSION

As duly justified in the Introduction, even though *C. elegans* has the IUCN conservation category of "Least Concern"[11], monitoring populations *in situ* will allow the development of long-term conservation strategies, specially under an uncertain panorama of global climate change that could lead to unexpected plagues, for example. This study is the first monitoring effort of the population of *Croton elegans*, an endemic species from Ecuador, and has included an estimate of the real population density of the species, along with data on Height, Cover, Neighborhood Index and Phenological Status. This information creates a baseline to assess the real conservation status of this endemic plant in the long-term.

One important variable to emphasize is plant density (on average, 50 individuals / 40 m^2 ; Figure 4). Indeed, the number of publications reporting plant density of *Croton* species is very scarce. For example, *Croton wagneri*, a plant also endemic to Ecuador, is reported to be locally abundant in the valley of Catamayo (Loja province, Ecuador)[27], whereas Aplet *et al.* (1994)[23] in Texas, USA, reports for *Croton alabamensis* a density of 50 individuals / 100 m^2 which is a much lower density than what is reported here for *C. elegans* when extrapolated to the same area (125 individuals / 100 m^2).

Correlational studies do not allow the unambiguous identification of the causal mechanisms that underlie clear patterns[28]. Nevertheless, detecting geographic variation

in a trait can be a first step towards proposing biological/ecological mechanisms that may account for it. In this study, the *C. elegans* population did not show a correlation between abundance and growing zone (Figure 4). Generally, elevation is one of the most important determinants of plant community composition and diversity through its influence on the length of the growing season, temperature, moisture availability, substrates, and soil fertility[29]—yet, the 100-m altitudinal gradient at San Eloy Hill is probably too small to show these effects. In other words, it can be argued that (1) there are optimal ecological conditions or similar environmental conditions for the development of *C. elegans* along the whole topographic gradient of San Eloy Hill; or (2) even if there were different ecological/environmental conditions between the hilltop and the hill base of San Eloy Hill, the *C. elegans* species seems not to be ecophysiologically affected (it seems to have a high degree of plasticity).

Figure 5 shows descriptive data about morphometry (Height and Cover), Neighborhood Index and Phenology Status obtained for the *C. elegans* population studied. The patterns observed will serve as baseline data for future monitoring studies of the *C. elegans* population at Yachay Botanical Garden, which may be particularly relevant in the face of global changes, such as global warming. For example, it could be argued that with increasing CO_2 concentrations, the biomass of many plant species (a function of height and cover) should increase[30]— it would be interesting to assess if this would also be the case of *C. elegans*. Regarding the Neighborhood Index data, they actually become more interesting when contrasted to other variables (see next paragraph about Figure 6). Finally, with respect to the data on Phenological Status, it should be noted that they can become the basis for future in-depth projects on the reproductive biology of this species; for now, at least, it is clear from the data that from June to November the species is fertile in the Yachay Botanical Garden area.

In general, individual plants with different neighborhoods experience different growth, size, survival, and reproductive conditions[31], which provide information for inferring the sign and strength of plant-plant interactions in field conditions. In this sense, the results suggest that plant-plant interactions (low and high Neighborhood Index) directly affect the *C. elegans* plant size (Height and Cover) in the population studied (Figures 6 and 8). This finding could be explained by the fact that any species exhibit intraspecific variation in most functional and phenotypic traits. Such variation, in turn, helps a species to deal with many biotic interactions. In the case of *C. elegans* plants growing under high

neighborhood conditions, it is evident that they must compete by light and nutrients, which may actually increase their size.

The *C. elegans* population at San Eloy Hill shows a negative correlation between Height and Elevation, and between Cover and Elevation (Figures 7 and 8). According to Bakhtiari et al. (2019)[32], two reasons have been put forward for plants to reduce growth at high elevation. First, a decrease in the general metabolic activity as a function of colder temperature inhibits photosynthetic rate and biomass production. Second, it has been proposed that because plants growing at higher elevations typically receive direct sunlight and higher ultraviolet radiation, and ultraviolet radiation destroys the auxins content at the apical shoots, and thus they tend to grow much slower than lowland plants. It would be interesting to carry out more samplings along the altitudinal range of San Eloy Hill, with the aim of discovering the altitude at which the plants start to significantly reduce their Height (the inflexion point).

In this study, there is a clear signal that plant density of *C. elegans* in the transects sampled relates to the Neighborhood Index (NI) values, at least those with values equal to 1 (low NI) or 2 (moderate NI) (Figure 9). This signal can be linked to the sign and strength of plant-plant interactions[33]. In this study, *C. elegans* seems to better develop in patches with a lower Neighborhood Index, because fewer individuals are recorded in patches with a high Neighborhood Index. In addition, this spatial local distribution was present at all altitudinal zones, which shows that inter-specific crowdedness deeply influences the population structure at the study area. According to Lara-Romero *et al.* (2017)[33], local neighborhood conditions have an effect in spatial position, growth, size, and reproduction of individuals. Focusing on neighborhood processes that affect the small-scale spatial structure of plant populations gives us insight into other relevant ecological processes, such as dispersal and plant-plant interactions[34]. These analyses also provide insight into plant fate along the demographic cycles of species, which is relevant given the common lack of datasets on spatiotemporal patterns of population dynamics[34]. In general, species that occupy xerophytic habitats do not have competitive abilities; for example, it has been documented that populations of *Croton glandulosus* show high density distribution when they do not have competition[35]. This is similar to what was observed for *C. elegans* in this study.

In conclusion, this study showed that *C. elegans* plants with lower mean Height occurred at higher altitudinal areas, where there was also less plant Cover. A Principal

Component Analysis indicated that morphometric variables were correlated with each other. In addition, there was evidence that Neighborhood Index, an indirect measure of potential inter-specific competition, was highly correlated to the abundance of *C. elegans* at the transect level. Results suggest that environmental factors correlated with the altitudinal gradient, together with potential inter-specific competition, may affect the population structure and morphometric characteristics of *C. elegans*.

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6. FIGURES

Figure 1. Location of Yachay Botanical Garden in Urcuquí, Imbabura, Ecuador.

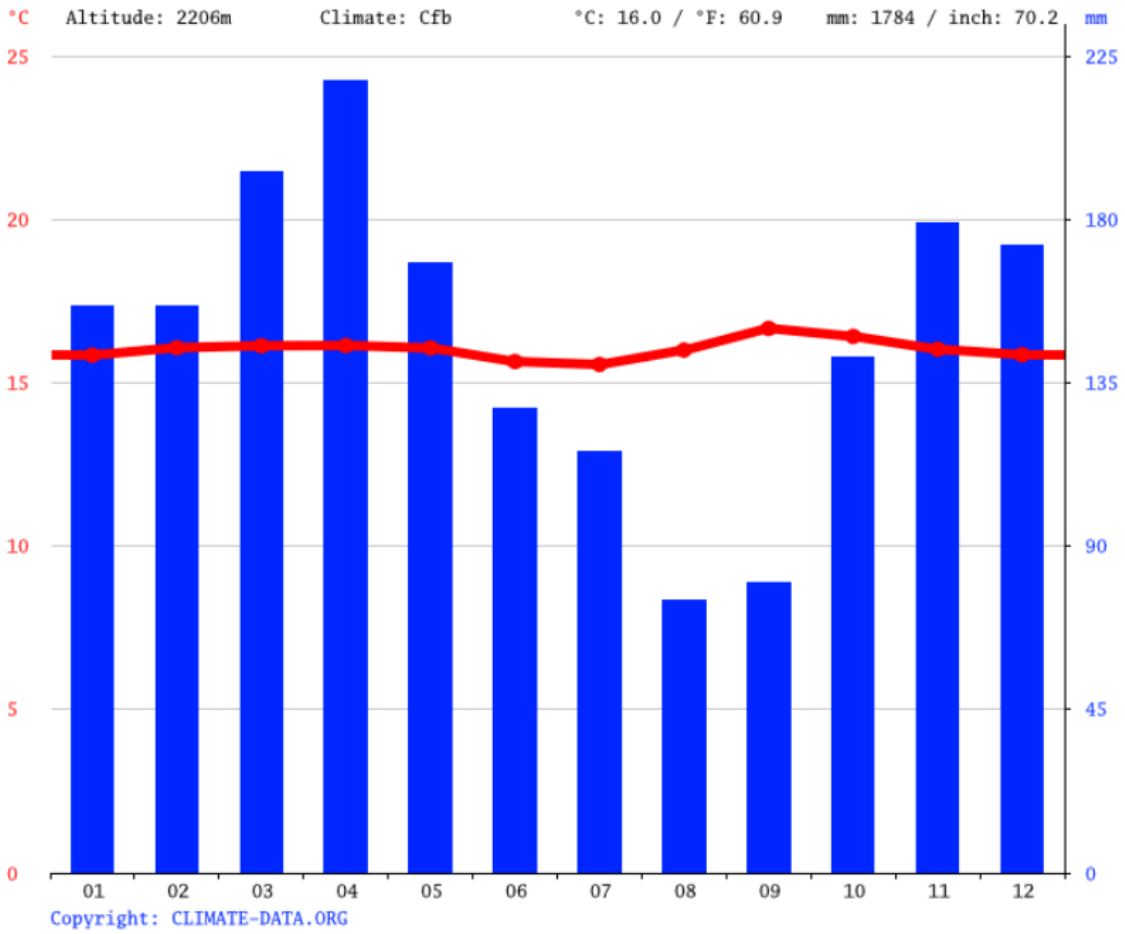


Figure 2. Climate graph of Ibarra, Imbabura, Ecuador. Data: 1991 - 2021 Min. Temperature 11°C, Max. Temperature 23°C, Precipitation / Rainfall 1784 mm. (Retrieved from Climate-Data.org).

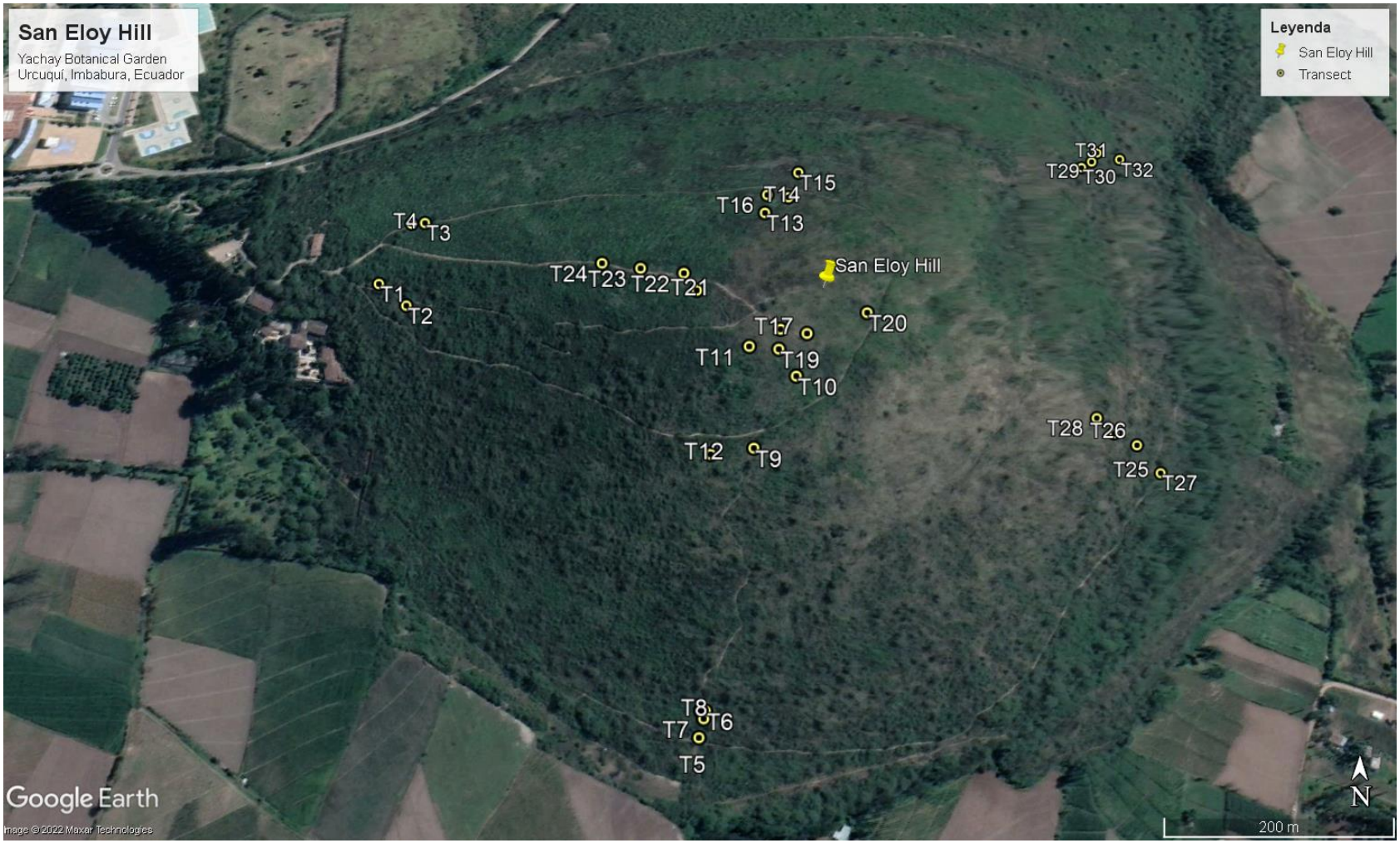


Figure 3. The San Eloy Hill study area at the *in situ* conservation area of Yachay Botanical Garden, Urcuquí, Ecuador, showing the geographic location of each transect.

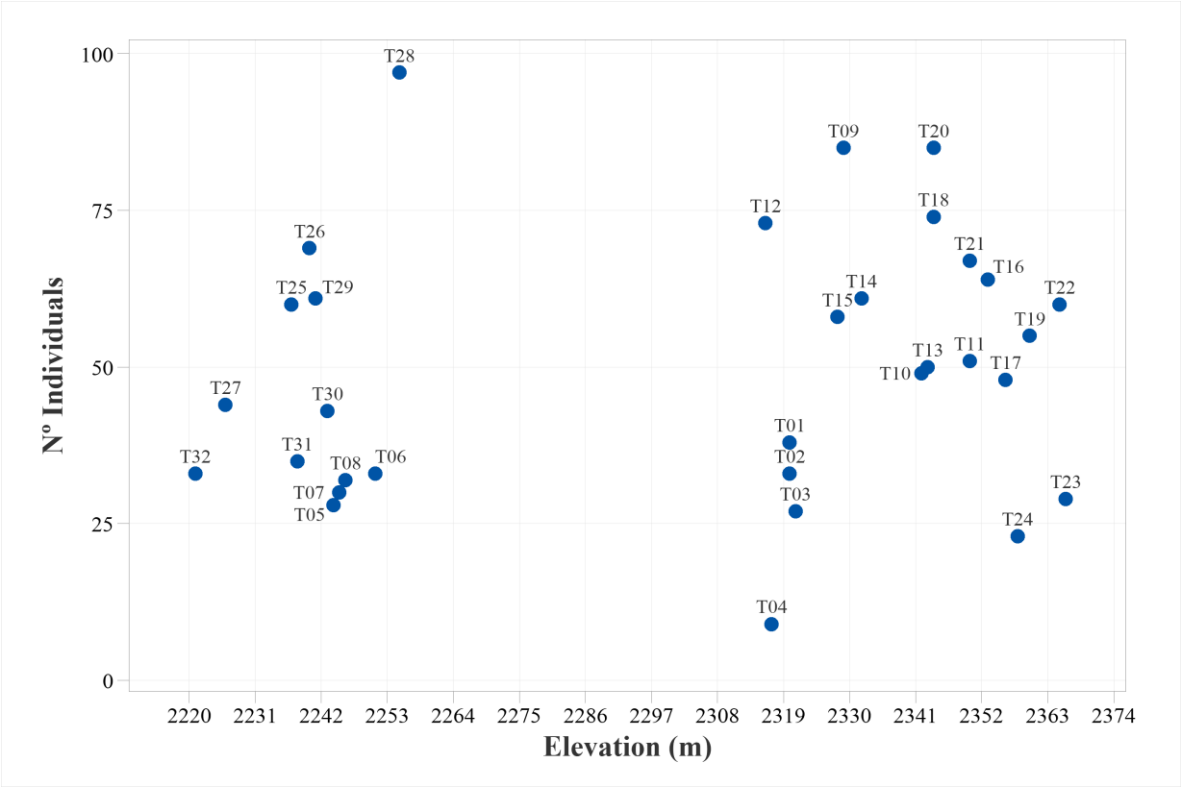
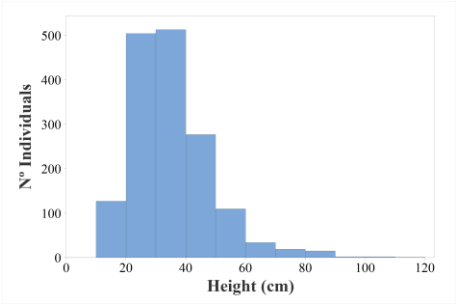


Figure 4. Abundance of *Croton elegans* individuals per transect along the elevation gradient at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Each symbol indicates one transect (*N* = 32 transects). There was no correlation between N° of individuals per transect and elevation (ρ = 0.14 , *P* = 0.4427).

a

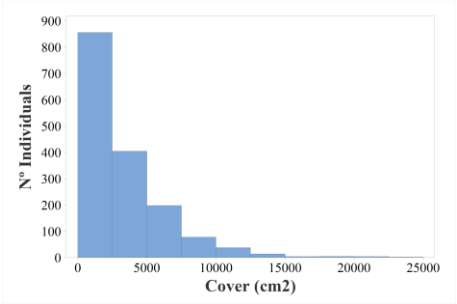
Height (cm)



Mean= 34.4
Median= 33
Mode= 30
Range= [10 – 118]
Standard deviation= 13.1
Kurtosis= 3.5
Skewness= 1.4

b

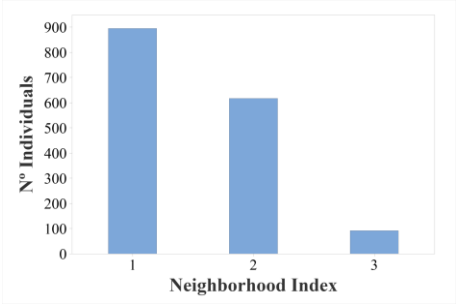
Cover (cm^2)



Mean= 3256.6
Median= 2328
Mode= 1979
Range= [44 – 23713]
Standard deviation= 3178.8
Kurtosis= 6.5
Skewness= 2.1

c

Neighborhood Index



Not applicable

d

Phenological Status

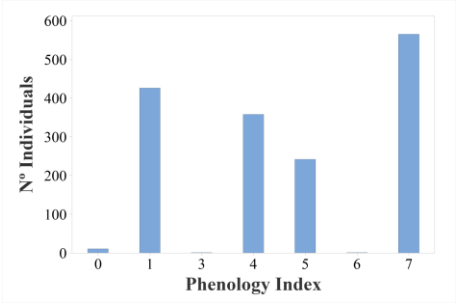


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Figure 5. Statistical summary of the data obtained from four population structure variables measured in a population of *Croton elegans* (*N* = 1604 individuals) sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Note that descriptive statistics were only calculated for the quantitative continuous variables of Height and Cover (no descriptive statistics were calculated for the other two variables, the indexes, which were either ordinal or categorical variables).

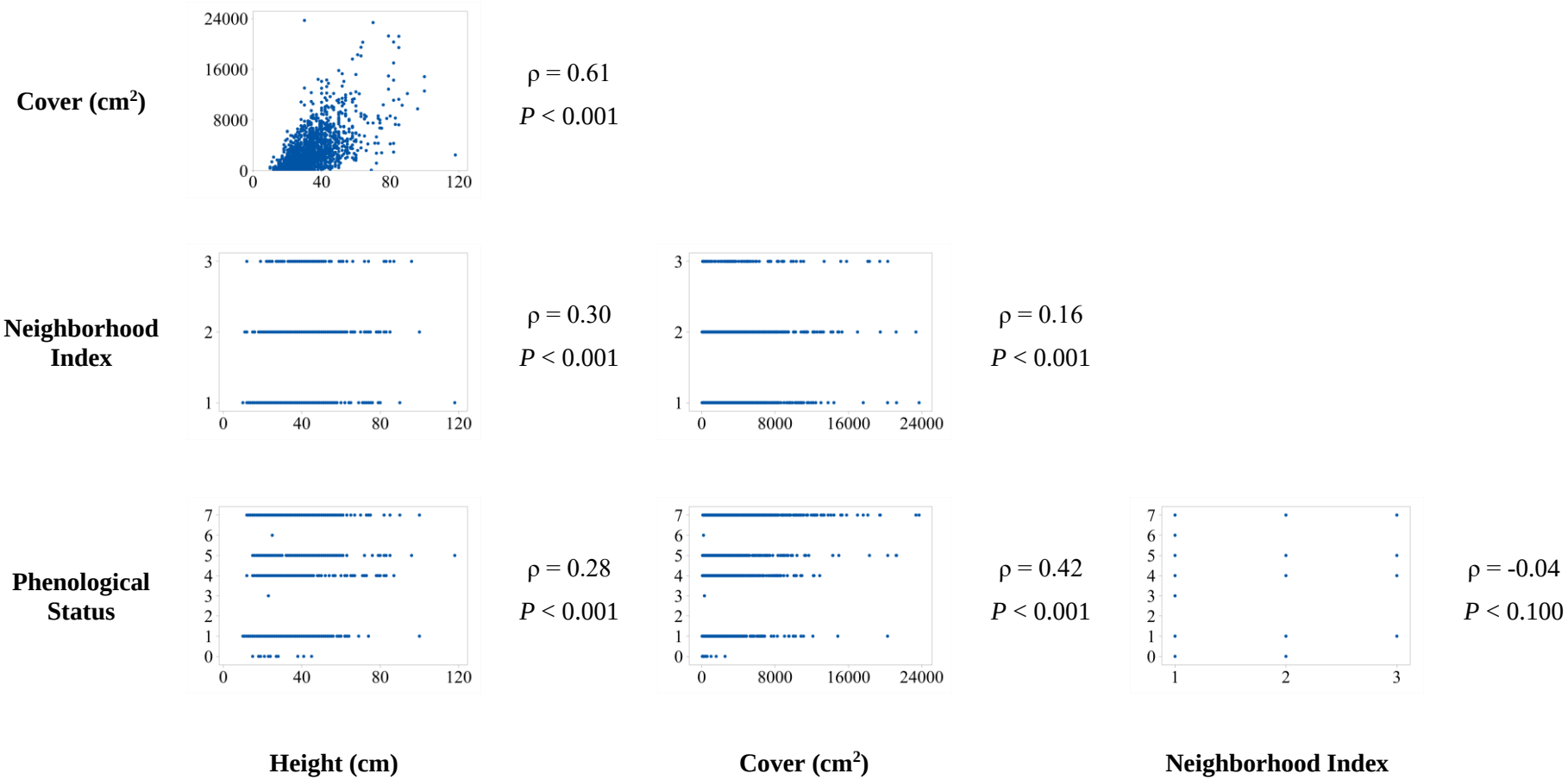


Figure 6. Scatter plots with Spearman correlations between the four population structure variables measured in a population of *Croton elegans* sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Each point represents one individual (*N* = 1604 individuals; but note many points overlap). ρ (rho) = Spearman correlation coefficient, *P* = probability value.

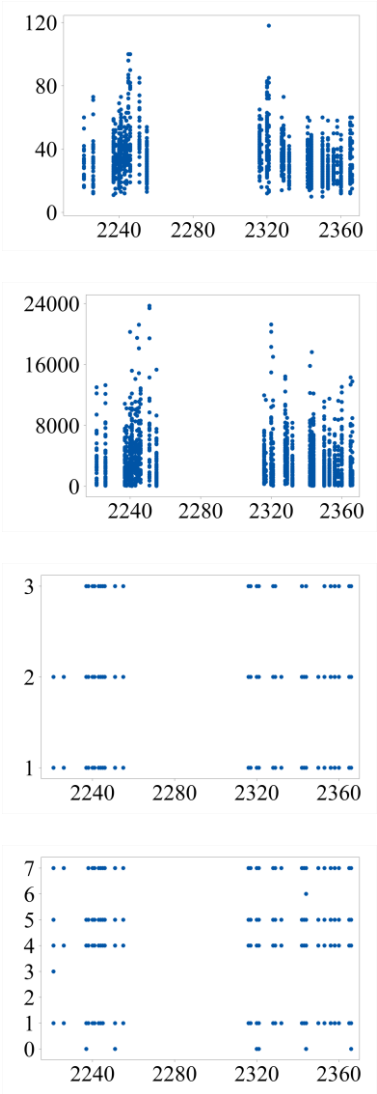


Figure 7. Scatter plots with Spearman correlations between elevation and the four population structure variables measured in a population of *Croton elegans* sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Each point represents one individual (N = 1604 individuals; but note many points overlap). ρ (rho) = Spearman correlation coefficient, P = probability value.

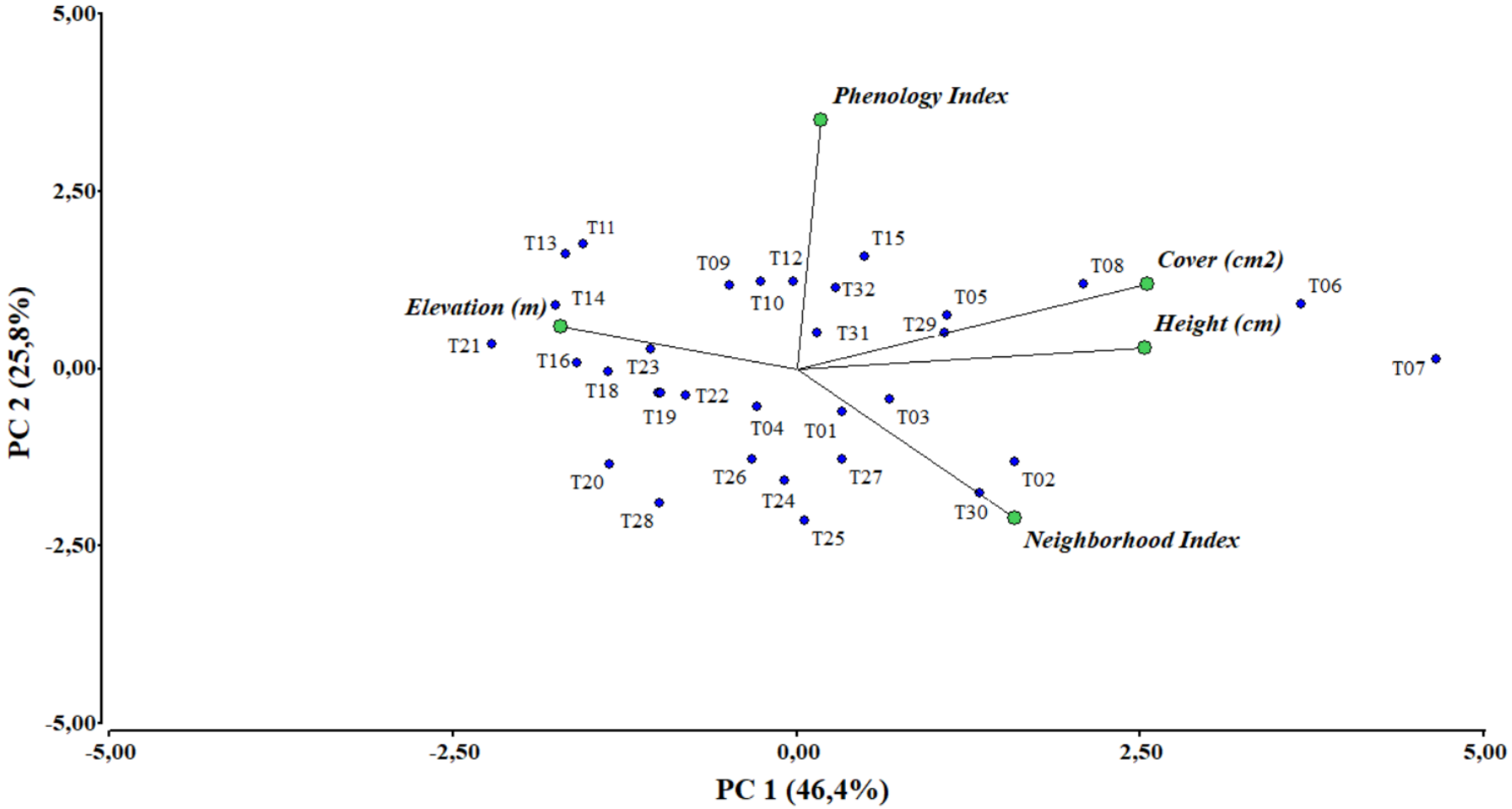
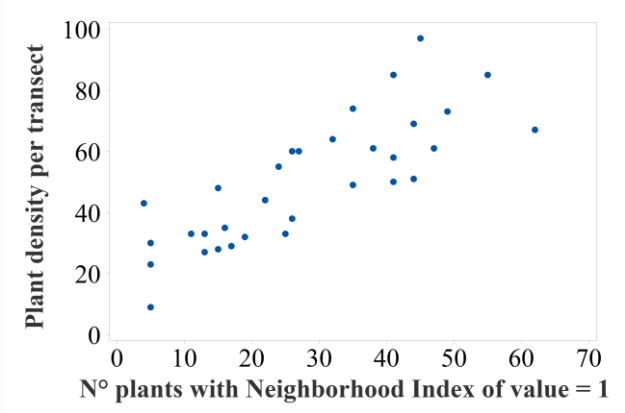


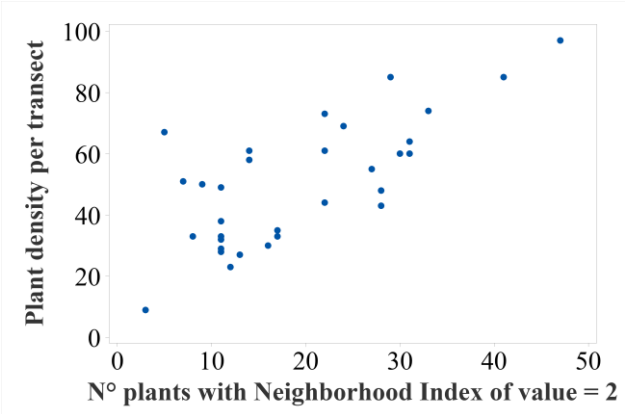
Figure 8. Principal Component Analysis (PCA) of five variables measured in a population of *Croton elegans* (*N* = 1604 individuals) sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Each blue point represents one transect (*N* = 32 transects).

a



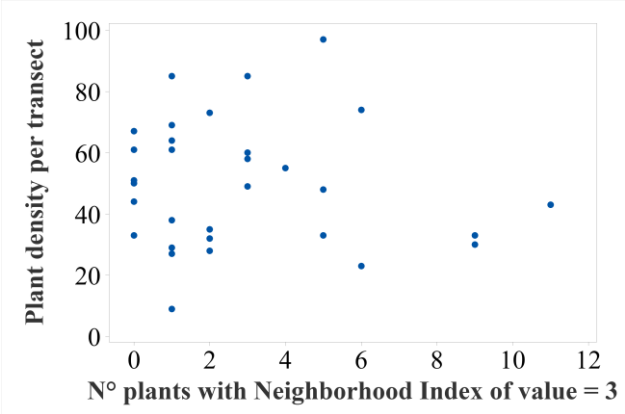
ρ = 0.85
P < 0.001

b



ρ = 0.62
P = 0.0002

c



ρ = -0,08
P = 0.673

Figure 9. Scatter plots with Spearman correlations between plant density and No. of plants with different values of the Neighborhood Index (1, 2 or 3) in a population of *Croton elegans* sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Each point represents one transect (*N* = 32 transects). ρ (rho) = Spearman correlation coefficient, *P* = probability value.

7. SUPPLEMENTARY MATERIAL

Table S1. Geographic location of the 32 transects established in this study to sample the population of *Croton elegans* at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Density of individuals per transect is also shown. Transects are ordered according to their elevation.

Transect	Elevation (m)	Latitude	Longitude	Nº Individuals
T32	2221	0.41936	-78.18156	33
T27	2226	0.41673	-78.1815	44
T25	2237	0.41691	-78.18169	60
T31	2238	0.41938	-78.18176	35
T26	2240	0.41697	-78.18188	69
T29	2241	0.41928	-78.18182	61
T30	2243	0.4192	-78.18192	43
T5	2244	0.41475	-78.18489	28
T7	2245	0.41487	-78.18486	30
T8	2246	0.41492	-78.18488	32
T6	2251	0.41492	-78.18485	33
T28	2255	0.41706	-78.18202	97
T12	2316	0.41653	-78.18484	73
T4	2317	0.41828	-78.18703	9
T1	2320	0.4178	-78.18722	38
T2	2320	0.41762	-78.187	33
T3	2321	0.41828	-78.18693	27
T15	2328	0.41868	-78.18423	58
T9	2329	0.41658	-78.18455	85
T14	2332	0.41843	-78.18431	61
T10	2342	0.41705	-78.18427	49
T13	2343	0.41827	-78.18448	50
T18	2344	0.41733	-78.1842	74
T20	2344	0.41752	-78.18378	85
T11	2350	0.41723	-78.18459	51
T21	2350	0.41761	-78.18495	67
T16	2353	0.41845	-78.18446	64
T17	2356	0.41735	-78.18438	48
T24	2358	0.41785	-78.18561	23
T19	2360	0.41722	-78.18439	55
T22	2365	0.41775	-78.18504	60
T23	2366	0.4178	-78.18534	29

Table S2. Spearman correlations between four population structure variables measured in a population of *Croton elegans* at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador, based on sub-sampling procedures in which only 100 individuals were randomly selected, in order to control for the artificial effect on *P*-values due to the extremely high sample size of the original analysis (*N* = 1604 individuals; see Figure 6). This procedure was repeated five times for each correlation conducted.

Sub-sample ID	ρ	*P*	*N*
Spearman correlation between Height (cm) and Cover (cm^2)			
1	0.66	< 0.001	100
2	0.67	< 0.0001	100
3	0.58	< 0.0001	100
4	0.57	< 0.0001	100
5	0.59	< 0.0001	100
Spearman correlation between Height (cm) and Neighborhood Index			
1	0.29	0.0037	100
2	0.43	< 0.0001	100
3	0.25	0.0126	100
4	0.35	0.0004	100
5	0.28	0.0044	100
Spearman correlation between Height (cm) and Phenology Index			
1	0.29	0.0032	100
2	0.26	0.0080	100
3	0.37	0.0002	100
4	0.41	< 0.0001	100
5	0.27	0.0058	100
Spearman correlation between Cover (cm^2) and Neighborhood Index			
1	0.21	0.0391	100
2	0.12	0.2475	100
3	0.27	0.0063	100
4	0.21	0.0363	100
5	0.16	0.1098	100
Spearman correlation between Cover (cm^2) and Phenology Index			
1	0.47	< 0.0001	100
2	0.39	0.0001	100
3	0.37	0.0002	100
4	0.54	< 0.0001	100
5	0.41	< 0.0001	100
Spearman correlation between Neighborhood Index (cm^2) and Phenology Index			
1	0.07	0.4972	100
2	0.02	0.8413	100
3	-0.11	0.2755	100
4	-0.01	0.9212	100
5	-0.22	0.0247	100

Table S3. Spearman correlations between elevation and the four population structure variables measured in a population of *Croton elegans* at San Eloy Hill. Yachay Botanical Garden. Urcuquí. Ecuador, based on sub-sampling procedures in which only 100 individuals were randomly selected, in order to control for the artificial effect on *P*-values due to the extremely high sample size of the original analysis (*N* = 1604 individuals; see Figure 7). This procedure was repeated five times for each correlation conducted.

Sub-sample ID	**ρ**	***P***	***N***
Spearman correlation between Elevation (m) and Height (cm)			
1	-0.27	0.0063	100
2	-0.24	0.0159	100
3	-0.21	0.0370	100
4	- 0.23	0.0216	100
5	-0.34	0.0005	100
Spearman correlation between Elevation (m) and Cover (cm^2)			
1	-0.13	0.1907	100
2	-0.18	0.0786	100
3	-0.17	0.0897	100
4	-0.26	0.0082	100
5	-0.21	0.0370	100
Spearman correlation between Elevation (m) and Phenology Index			
1	0.10	0.3386	100
2	0.08	0.4375	100
3	0.17	0.0909	100
4	0.17	0.0822	100
5	0.09	0.3854	100
Spearman correlation between Elevation (cm) and Neighborhood Index (cm^2)			
1	-0.02	0.8281	100
2	0.05	0.6009	100
3	-0.07	0.4687	100
4	-0.07	0.4682	100
5	-0.14	0.1886	100

CHAPTER II

CHAPTER II. Metabolic expression of *Croton elegans* Kunth in different microhabitats at San Eloy Hill, Yachay Botanical Garden: preliminary phytochemical screening, total phenolic content and total flavonoid content

ABSTRACT

Croton elegans Kunth is a common, endemic and medicinal shrub that is widely distributed in the dry interandean valleys of Ecuador, including that of San Eloy Hill, at Yachay Botanical Garden (YBG), Urcuquí, Imbabura. In this study, I asked how metabolic expression in leaves of *C. elegans* may change depending on location along a short altitudinal gradient and on interspecific plant-plant competition (spatial crowdedness) estimated via a "Neighborhood Index" (NI). In this context, I randomly collected three independent plant replicates for each of the following combinations: Hilltop – High NI; Hilltop – Low NI; Hill base – High NI; and Hill base – Low NI. Once leaf methanolic extracts were obtained for each replicate, a general qualitative phytochemical screening was conducted, complemented by a quantitative assessment of total phenolic content and total flavonoid content. Results showed that all methanolic extracts, independently of altitude and neighborhood condition, had the same constituent of secondary metabolites, and that neither phenols nor flavonoids showed significant differences in their content. In conclusion, the very short altitudinal range of San Eloy Hill at YBG did not seem to considerably affect the qualitative and quantitative expressions of secondary metabolites of the local *C. elegans* population.

Keywords: Secondary metabolites, altitudinal gradient, neighborhood index, *Croton elegans*

1. INTRODUCTION

Ecuador is considered as one of the countries with the highest biodiversity per unit area, ranked sixth in the world[1,2]. It also possesses high plant endemism[3], currently represented by ~6000 species, i.e. ~30% of Ecuador´s total number of native species (C. Ulloa, pers. comm.). One of the areas with higher endemism are the dry forests; among them, the interandean dry forests are mostly located in the provinces of Imbabura, Pichincha, Azuay and Loja[3,4].

In Ecuador, the use of medicinal plants is a widespread, orally transmitted, practice, specially by the indigenous population that use ancestral knowledge[5–7] to cure infectious diseases caused by parasites (incl. bacteria and viruses) and related to skin diseases, inflammations, tumors, bronchial conditions, fever, pain, among others[3,7]. Numerous studies report ethnobotanical use of Ecuadorian plant species[6,8–10], but scientific information on their phytochemical or biological activity is yet insufficient. The medicinal properties of these plants are due to the phytochemical constituents---primary and secondary metabolites---that cause definite pharmacological actions on the human body[11,12]. Primary metabolites are vital for the proper growth of plants[13] and include amino acids, carbohydrates, proteins, chlorophyll, among others[11]. Secondary metabolites, on the other hand, do not play any role in the internal economy of a plant[13] and include alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds, among others[5,11,14]. The majority of these secondary metabolites are known to possess valuable therapeutic activities such as insecticidal, antibacterial, antifungal, antiplasmodial, anti-inflammatory, antiviral and antioxidant activities[11,14].

Various biotic factors such as plant age, soil microbiota, grazing, competition and nutritional status have shown to have an impact in the production and concentration of secondary metabolites[15–17]. Also, abiotic (environmental) factors like precipitation, temperature, relative humidity, soil properties, wind speed, growing season length, and the radiation intensity can affect secondary metabolites production, and have been reported to differ between sites of low vs. high altitudes[15,17,18]. Among these variables, high solar radiation at higher altitudes has been mainly presented and discussed as one of the main factors that impact secondary metabolite profiles[17].

In this study, I focus on the phytochemistry of *Croton elegans* Kunth, a shrub endemic to the dry interandean vegetation of Ecuador, locally known with the common name "Mosquera"[19]. According to folk medicine, *C. elegans* is used in the treatment of

toothache, wounds, tonsillitis, warts, neuralgia, rheumatism, gout and bronchitis[8]. Despite the widely studied phytochemicals in the *Croton* genus---rich and diverse in chemical constituents such as active alkaloids, flavonoids and terpenoids[20,21---]analysis of the qualitative and quantitative phytochemical properties of *C. elegans* have not been reported previously in literature. Several *Croton* species have been used traditionally as natural remedies by indigenous cultures around the world, due to its wide range of pharmacological properties, including weight loss and treatment against cancer, constipation, diabetes, digestive problems, dysentery, external wounds, ulcers, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria and pain [20–22]. Furthermore, several species of *Croton* are aromatic, indicating the presence of volatile oil constituents such as monoterpenes and sesquiterpenes[20,22,23].

Among the Ecuadorian native species of *Croton* whose phytochemistry has been studied, we can highlight: 1) *Croton lechleri* Müll. Arg., a tree native to the Amazon of Ecuador, Peru and Bolivia, whose blood-reddish latex and its taspine alkaloid have proven wound healing, anti-inflammatory and anticancer properties[20,24,25]; 2) *Croton pycnanthus* Benth., a near threatened shrub endemic to the northern Ecuadorian Andes, has demonstrated, by an experimental assay using mouse bone marrow-derived macrophages (BMMs), its potential therapeutic effect on osteoporosis and osteolytic diseases, given its inhibitory effects on osteoclast differentiation without cytotoxicity[26]; 3) *Croton lobatus* L., a herb occurring in the Ecuadorian provinces of Guayas and Manabí, traditionally used to treat malaria, has shown anti-plasmodial activity towards the 3D7 chloroquine sensitive and the K1 chloroquine resistant strains of *Plasmodium falciparum*[27]; and 4*) Croton schiedeanus* Schlecht, a tree known to occur in the Ecuadorian provinces of Esmeraldas, Guayas, and Los Ríos, is known to decrease blood pressure in anaesthetized spontaneously hypertensive rats through an antihypertensive and vasodilatory effect—an experimental result that agrees with its traditional use against hypertension[28]. These interesting medicinal properties are most probably applicable to many other *Croton* species yet to study in Ecuador. Indeed, let´s recall that *Croton* is the second largest genus of the Euphorbiaceae family and has approximately 1300 species worldwide distributed throughout tropical and subtropical regions, from which 38 occur in Ecuador[20,21,29].

In this general context, using samples from 12 *C. elegans* individuals collected from different elevations and neighborhood conditions at San Eloy Hill, Urcuquí, Ecuador, I defined the following objectives and hypotheses for this study:

1.1. Objectives

- To characterize the phytochemicals constituents in the species *C. elegans* qualitatively.
- To determine the total phenolic and total flavonoid content of extracts of *C. elegans* in different elevation gradients.
- To identify the extent to which environmental factors such as elevation and neighborhood index affect the phytochemical contents among the samples.

1.2. Hypotheses

- The phytochemical profile varies according to the elevation of the *C. elegans* growth site.
- The phenols and flavonoids content differ between *C. elegans* individuals grown in different elevation gradients.

2. MATERIALS AND METHODS

2.1. Study area

The study was conducted in February 2022, at San Eloy Hill within the *in situ* conservation area of Yachay Botanical Garden (YBG), in Urcuquí, northern Andes of Ecuador (geographic coordinates at the hilltop: 0°25'03" N 78°11'04" W). The conservation area at San Eloy Hill covers ca. 0.6 km^2 and has an altitudinal range from ca. 2200 to 2345 m. The native ecosystem that naturally occurs at YBG is officially classified as "bosque y arbustal semideciduo de los valles del norte" (semideciduous forest and shrubland of the northern valleys), also known as dry interandean valley vegetation (Ministerio del Ambiente del Ecuador, 2013)[30].

Regarding the climate, the most trustful data in the area is that from Ibarra [1991-2021; Climate-Data.org[31]], the capital province, located at 10 km from the study area in Urcuquí. Mean interannual monthly temperature varies from 15.6 °C (June/July) to 16.7 °C (September). Minimum temperatures tend to occur in August (ca. 11 °C) while highest

temperatures tend to occur in September (ca. 23 °C). Average yearly rainfall is ca. 1700 mm/year, with monthly rainfall ranging from 75 mm (August) to 218 mm (April). Historically, the dry period starts around June, intensifying in August/September.

2.2. Sampling design

First, four plant groups were delimited using the following two FACTOR conditions, each with two levels (Table 1): Factor 1 was "Location in the hill", with hilltop (2320 – 2370 masl) and hill base (2220 – 2255 masl) levels; Factor 2 was "Neighborhood index" with high and low levels. The "Neighborhood index" is hereby defined as a qualitative categorical assessment (possible values: 1, 2 or 3) that estimates how dense the vegetation surrounding a plant is, where "1" means low plant density around a plant, and "3" means high plant density around a plant.

In each of the four groups defined, three plants of *Croton elegans* were randomly selected (total = 12 plants; Table 2) within a range of plant height (30 – 70 cm) and plant cover (5.200 – 14.200 cm^2) chosen to avoid sampling individuals from a size interval in which plants can be either too small or too scarce. Plant Height was defined as the distance from soil level to the taller apical meristem (non-extended). Plant Cover was defined as the calculated area of an imaginary ellipse covering the whole plant canopy (therefore, two measurements were taken: the length of longest canopy axis and, at 90° from it, the length of the short canopy axis).

For each plant, several mature relatively intact sun leaves were collected, while avoiding extensive damage to the plant. Leaves were collected during February 2022 and temporarily stored in a labeled paper bag. Once in the lab, leaves were washed with distilled water to remove dust or insects, and then oven dried at 45ºC for three days. Every group of leaves, belonging to a given plant, is hereby referred to as a "sample", even though statistically speaking it is really a "sampling unit" (the statistical sample would really be represented by the three plants in each group).

Plant identification was confirmed at confirmed at the National Herbarium of Ecuador (QCNE), and a voucher specimen (Sánchez-García 0001) deposited there.

2.3. Methanolic extraction

To assess qualitatively the secondary metabolites contained in each sample (N = 12 plants), a maceration procedure was repeated three times (Figure 1). In the first maceration, ca. 10 g of chopped dry plant material was mixed with 170 mL of methanol in an Erlenmeyer flask. This plant-solvent mixture was placed under constant stirring at 300 revolutions per minute (rpm) at room temperature for 24 hours. This mixture was then gravity-filtered, and the extract stored in a glass bottle (funnel, filter paper, and glass bottle were all washed with the solvent before filtering). For the second maceration, the plant residue of the first maceration was mixed with 100 mL of methanol in an Erlenmeyer flask, and the stirring and filtering procedures repeated. The extracts from the first and second macerations were then mixed in the same glass bottle. For the last and third maceration, the plant residue of the second maceration was mixed with 70 mL of methanol in an Erlenmeyer flask, and the stirring and filtering procedures repeated. The extract from this last maceration was also stored in the same glass bottle, as before.

To remove the solvent (methanol), the extracts were evaporated under reduced pressure for approximately 4 hours, using a rotary evaporator set at a temperature of 35 ºC and a rotation speed of 40 rpm. The extracts were transferred to glass vials and lyophilized by one day, to dryness. These freeze-dried ***methanolic extracts*** were stored in a refrigerator at 4° C, protected from sunlight, for future use in the phytochemical and antimicrobial analyses.

The extraction yield (%) of dry plant material was calculated by the following Equation 1:

$$Extraction\ yield\ (\%) = \frac{Dry\ extract}{Dry\ plant\ material}\ x\ 100 \quad (1)$$

To assess if there was a relation between the extraction yield and elevation gradient a Pearson's correlation was conducted, using InfoStat Software.

2.4. Qualitative phytochemical screening

A qualitative phytochemical screening allows the detection (presence/absence) of primary metabolites (*e.g.*, proteins, carbohydrates, lipids) and secondary metabolites (*e.g.*, alkaloids, flavonoids, phenols, saponins, steroids, glycosides, cardiac glycosides, tannins, terpenoids, among others) that a plant extract contains. Secondary metabolites

can become potential sources of bioactive agents for the synthesis of useful drugs with specific biological activity. In this study, the different phytochemical tests followed standard procedures, with minor modifications (Ref. 62–73). The protocols followed for each of the tests are described below. Just before the start of any test, the freeze-dried (lyophilized) methanolic extract being tested was dissolved in methanol or water, depending on the test protocol, using a 1:1 (w/v) proportion (for example, 10 mg extract: 10 mL solvent; or 50 mg extract: 50 mL solvent). In the protocols below, we refer to these solutions as ***alcoholic or aqueous test solutions***.

2.4.1. Tests for alkaloids detection

For the Wagner's test[32–35], 2 drops of Wagner's reagent were added, along the sides of the test tube, to 1 mL of an alcoholic test solution. The formation of a reddish or brown precipitate indicates the presence of alkaloids.

For the Mayer's test[32,33,36–39], 3 drops of Mayer's reagent were added along the sides of the test tube to 1 mL of an alcoholic test solution. The formation of a creamy or yellow precipitate indicates the presence of alkaloids.

For the Dragendroff's test[32,33,38], 1–2 mL of Dragendroff's reagent were added to 1 mL of an alcoholic test solution. A reddish-brown precipitate indicates the presence of alkaloids.

For the Iodine test[32,35,39], 3 drops of iodine solution were added to 3 mL of an alcoholic test solution, which was then heated gently. The formation of a blue color when the solution is heated indicates the presence of alkaloids.

2.4.2. Tests for flavonoids detection

For the Alkaline reagent test[32,36,40], 2 mL of 2% NaOH solution were added to 1 mL of an alcoholic test solution, resulting in an intense yellow solution. The presence of flavonoids is confirmed by the change to a colorless solution when 5 drops of diluted HCl are added.

For the Shinoda's test[32,39,40], few fragments of magnesium ribbon (enough to fill the tip of a small spatula lab) were added to 5 mL of an alcoholic test solution, followed by dropwise addition of concentrated HCl. The presence of flavanol glycosides is indicated by the formation of a pink to crimson colored solution.

For the concentrated H_2SO_4 test[32,41], 0.5 mL of concentrated H_2SO_4 were added to 1 mL of an alcoholic test solution. An orange color indicates the presence of flavonoids.

2.4.3. Tests for phenolic compounds detection

For the Iodine test[32,36], 4-5 drops of diluted iodine solution were added to 1 mL of an alcoholic test solution. The formation of a transient red color indicates the presence of phenolic compounds.

For the Ferric chloride test[32,36,38], 4-5 drops of 5% ferric chloride solution were added to 1 mL of an aqueous test solution. The presence of phenolic compounds is indicated by the formation of a dark green or bluish black color.

2.4.4. Test for Tannins detection

For the Braymer's test[32], 3 mL of distilled water and 5 drops of 10% of ferric chloride solution were added to 1 mL of an alcoholic test solution. A blue or green color indicates the presence of tannins.

For the 10% NaOH test[32,36], 4 mL of 10% of NaOH solution were added to 1 mL of an alcoholic test solution, and then well-shaken. The formation of an emulsion in the solution indicates the presence of hydrolysable tannins.

2.4.5. Test for Saponins detection

For the Foam test 1[32,36,38], 2 mL of distilled water were added to 0.5 mg of dry methanolic extract, and then shaken vigorously. The presence of persistent foam for 10 minutes indicates the presence of saponins.

For the Foam test 2[32,42], 5 mL of distilled water were mixed with 0.5 mg of dry methanolic extract, after it was shaken vigorously, and for last heated to boiling. The appearance of creamy miss of small bubbles indicates the presence of saponins.

For the $NaHCO_3$ test[32,43], 0.5 mL of potassium bicarbonate solution and 0.5 of distilled water were added to 1 mL of an alcoholic solution, and then shaken vigorously. The presence of a stable honeycomb like froth indicates the presence of saponins.

2.4.6. Test for carbohydrates detection

For the Molish's test[32], to 2 mL of an alcoholic solution, 3 drops of alcoholic 1-napthol and 1 mL of H_2SO_4 along the sides of test tubes (carefully) were added. The formation of a violet ring indicates the presence of carbohydrates.

For the Resorcinol test[32], to 2 mL of an aqueous solution were added a few crystals of resorcinol, equal volume of concentrated HCl (2 mL), and then is heated. The formation of a rose color indicates the presence of ketones.

2.4.7. Test for Glycosides detection

For the Borntrager's test[32], 2 mL of an alcoholic solution were treated with 3 mL chloroform, after it was shaken well until the chloroform layer is separated, and then 1 mL of 10% of Ammonia solution was added. The presence of glycosides is indicated by the presence of a pink color solution.

For the Aqueous NaOH test[32,44], 1 mL of distilled water and 5 drops of aqueous NaOH solution were added to 0.5 mg of dry methanolic extract. A yellow color indicates the presence of glycosides.

For the concentrate H_2SO_4[32,39], 2 mL of glacial acetic acid, 2 drops of 5% of $FeCl_3$, and 3 drops of concentrated H_2SO_4 (carefully) were added to 2 mL of an alcoholic solution. The presence of a brown ring indicates the presence of glycosides.

2.4.8. Test for Cardiac Glycosides detection

For the Keller-Killani test[32,36], 1 mL of an alcoholic solution was treated with 1.5 mL of glacial acetic acid, 3 drops of 5% of ferric chloride, and then 1 mL of concentrated H_2SO_4 along the side of test tube (carefully). The formation of a blue color solution in the acetic acid layer indicates the presence of cardiac glycosides.

2.4.9. Test for Proteins and Amino acids detection

For the Ninhydrin test[32,33], 4 drops of ninhydrin solution (10 mg ninhydrin plus 200 mL acetone) were added to 2 mL of an alcoholic solution. The formation of a purple color solution indicates the presence of amino acids.

2.5. Quantitative phytochemical screening of phenols and flavonoids

2.5.1. Total Phenolic Content (TPC)

Total phenolic content of each sample was determined in triplicate by the Folin-Ciocalteu procedures according to the method of Bray and Thorpe (1954)[45] and modified by Sepahpour, *et al.* (2018)[46] with minor changes.

Briefly, dried extracts were prepared at 1.0 mg/mL concentration in methanol and diluted to obtain a concentration of 0.5 mg/mL. Folin-Ciocalteu reagent was diluted in a 1:10 (v/v) ratio with distilled water, 7.5% wt potassium carbonate (K_2CO_3. w/v) solution was prepared. Gallic acid standard solutions were diluted with distilled water and prepared at 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 mg/mL concentrations. Then, each test solution (extracts, standard, and blank) of 300 uL were mixed with 1500 uL of the 10% wt Folin-Ciocalteu reagent and incubated for 5 minutes at room temperature in the dark. After that, 1200 uL of 7.5% wt potassium carbonate solution were added. The mixtures were allowed to stand in the dark for 30 minutes. The absorbance of the resulting solution was recorded at a wavelength of 765 nm using a Zuzi Model 4211/50 spectrophotometer (ROGO-SAMPAIC, France) against a blank. The blank consisted of all reagents and solvents without sample extract or standard. TPC values of each individual extract sample were determined by comparison with the calibration curve prepared with a series of gallic acid standards, and the results expressed in mg of gallic acid equivalents per g dry weight extract (mg GAE/g DWE). The analysis were conducted in triplicate and the results expressed as mean ± standard error (SE) (Table 5).

2.5.2. Total Flavonoid Content (TFC)

Total flavonoid content of *C. elegans* extracts was determined by the use of a modified colorimetric method reported by Jia, *et al.* (1999)[47] and modified by Al-Nemari, *et al.* (2020)[48] with minor changes.

First. dried extracts were prepared at 1.0 mg/mL concentration in methanol and diluted to a concentration of 0.3 mg/mL, 5% (w/v) sodium nitrite ($NaNO_2$), 10% (w/v) aluminum chloride ($AlCl_3$), and 4% (w/v) sodium hydroxide (NaOH) were prepared with distilled water. Quercetin dissolved in 96% ethanol was used as the standard at concentrations 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 1.00 mg/mL. After, an aliquot of 0.250 mL extract (0.5 mg/mL) was mixed with 1 mL of distilled water. Sodium nitrite (5%, wt, 0.075 mL) was added to the mixture and incubated for 5 minutes in the dark. Then, 10% aluminum chloride (0.15 mL) was added and incubated for 6 minutes. Finally, 4% sodium hydroxide (0.50 mL) was added, and the volume was adjusted to 5 mL with distilled water. The mixture was shaken carefully and then incubated for 15 minutes. Absorbance was read at 415 nm using a Zuzi Model 4211/50 spectrophotometer (ROGO-SAMPAIC, France) against a blank. The blank was prepared using all reagents and solvents without

sample extract or standard. The total flavonoid content was reported as quercetin equivalents in mg per g of dry weight extract (mg QE/g DWE), using a standard calibration curve. The analysis were conducted in triplicate and results are expressed as mean ± standard error (SE) (Table 5).

2.5.3. Correlations among TPC, TFC and factor variables (from the research design)

Using the statistical program InfoStat, the correlations between total phenolic, flavonoid content and factor variables (position in the hill and neighborhood index) were analyzed.

3. RESULTS

3.1. Determination of extraction yield

The percentage yield of the dry crude extracts obtained from each sample by the maceration extraction technique, using methanol as solvent, was calculated according to equation 1; the values are presented in Table 3. It was observed that plant extracts located in the hilltop obtained the highest yield with 19.9 % and 18.7 % the TL-2 and TH-2 sample, respectively. Likewise, TL-3 sample located at the hilltop presented the lowest yield with 11.7 %, followed by the BH-1 sample located in hill-base with 13.8 % yield. The yield of the extracts did not present a significant difference in any of the study variables, which reveals that the extraction yield obtained is not influenced by the location in the hill and/or by the neighborhood index.

3.2. Qualitative phytochemical screening of *C. elegans*

Phytochemical screening of methanolic extract of 12 *C. elegans* (N = 3 per groups, 4 groups) leaf samples collected from San Eloy Hill was carried out using various chemical assays in order to identify either the presence or absence of secondary metabolites such as phenolic compounds, flavonoids, alkaloids, glycosides, saponins, tannins, proteins and amino acids.

The results of these phytochemical analysis are shown on Table 4. It indicates that using Alkaline reagent, Shinoda, and Concentrate H_2SO_4 tests, flavonoids were present in all the samples evaluated. Braymer and 10% NaOH tests gave a negative result for tannins in all samples, except in TL-4, TL-10 and TL-16 where it shown a possible

presence with the Braymer test. Phenolic compounds, tested using Iodine and Ferric chloride reagents, were present in all samples. Furthermore, saponins evaluated with three different tests (Foam test 1, Foam test 2, and $NaHCO_3$) yielded negative results for all samples. Molish test indicated the presence of carbohydrates in the samples, while Resorcinol test showed its absence. In addition, glycosides were present using Concentrate H_2SO_4, all the opposite with Borntrager and Aqueous NaOH tests that depicted negative results. Keller-Killani test showed the presence of cardiac glycosides. Proteins and amino acids were absent in all samples according to ninhydrin test.

3.3. Quantitative analysis of *C. elegans*

3.3.1. Total phenolic content and total flavonoid content

Total phenolic content and total flavonoid content were determined from the calibration curves of gallic acid ($Y = 8.9586x - 0.1619$, $R^2 = 0.9993$), and quercetin ($Y= 0.9333x + 0.0023$, $R^2 = 0.99949$), respectively. The total phenolic and total flavonoid contents among the different methanolic extracts of *C. elegans* leaves collected in San Eloy Hill (3 samples per group) can be seen in Table 5, expressed as % of the reference compound of dry weight extract (DWE). The results showed that the amount of TPC extracted was between 113.62 – 155.22 mg GAE/g DWE. The TPC (155.22 mg GAE/g DWE) was the highest in TL-2 sample followed by BL-3 (153.58 mg GAE/g DWE) and BH-2 (143.61 mg GAE/g DWE) samples. While the amount of TFC extracted was between 177.39 – 329.77 mg QE/g DWE. The highest TFC was found in TL-2 (329.77 mg QE/g DWE) sample followed by BH-3 (297.63 mg GAE/g DWE) and BH-2 (278.58 mg GAE/g DWE) samples. In general, there was major content of flavonoids than phenols in all samples evaluated (Figure 3).

There was no significant difference between TPC or TFC and the factor 1 (locations in the hill) with the factor 2 (neighborhood index). Besides, from the Figure 2 we can see that flavonoid has a significant correlation with total phenolic content ($r = 0.87$. $P < 0.001$), therefore, the level of total phenolics depends mainly on flavonoids.

4. DISCUSSION

4.1. Determination of extraction yield

The successful prediction of botanical compounds from plant material is mostly dependent on the type of solvent (affinity with the compound) used in the extraction procedure. Additionally, depending on the plant materials, the nature of the bioactive compound present also varies[49]. Methanol[50] is a universal solvent for phytochemical investigation because it is an unselective solvent; therefore, it allows the extraction of the majority of chemical components from the plant (e.g., alkaloids, anthocyanins, terpenoids, saponins, tannins, lactones, xanthoxylines, diterpenes, triterpenes, flavones, phenones, and polyphenols). Under those circumstances, the extraction yield presented in Table 3 is a related measure of the solvents efficiency in extracting specific components from the plant material evaluated. However, is recommended probe different solvents and extraction techniques to obtain more complete profile of secondary metabolites in *C. elegans* leaves.

4.2. Qualitative phytochemical screening of *C. elegans*

Plants used in traditional medicine represent a vast resource for the discovery and investigation of new drugs[48]. *C. elegans* has been used in traditional medicine in Ecuadorian cities, but the chemical and pharmacological characterization lies behind other species from the same genus, such as *C. lechleri*. In phytochemistry, one of the most critical challenges faced by researchers is that a single plant contains a lot of bioactive compounds.

Secondary metabolites (SM) are compounds that have no fundamental role in the maintenance of life processes in the plants, but they are important for the plants to interact with its environment for adaptation and defense against herbivores and pathogens[14], and have been defined as compounds whose biosynthesis is restricted to certain groups of plants[51]. This possesses specific enzymes capable of synthesizing distinctive metabolites only under suitable conditions[52]. Thus, these compounds often differ among individuals of the sample plant population in terms of their quantity and types.

Besides, altitudinal variation of secondary profiles of higher plants is not well investigated so far and numerous abiotic and biotic factors change with the elevation of the natural growing site[53]. These factors include precipitation, mean temperature, daily

thermal amplitudes, soil fecundity, wind speed, temperature extremes, atmospheric pressure, length of the vegetation period and radiation intensities[53].

In the present work, a first report of qualitative phytochemical screening of *C. elegans* leaves is presented, which allows to elucidate the biological activities and to examine possible differences in metabolite production according to samples collected in different positions in hill and with different neighborhood index.

The results obtained (Table 4) indicated the presence of flavonoids, phenolic compounds, carbohydrates, and glycosides in all samples (N = 3 per groups, 4 groups) evaluated, indicating the non-existence of a difference in the expression of SM. This may be due to the fact that the evaluated species were growing under similar environmental conditions, so the presence of all classes of metabolites evaluated could be related to their genetic characteristics, and the absence of certain compounds could be due to genetic factors since they do not possess the enzymes necessary for the production of these compounds[14]. Although, there are studies that argue that UV-B radiation affects the secondary metabolism of plants growing in high altitudes as a protective response against damage from excessive radiation in plants[53,55]. In this case, there are not difference between plants in the hilltop or hill base because the altitude difference between the plant samples from one end to the other only varies 125 meters, and this causes climatic and soil characteristic to be similar at the study zone.

On the other hand, according to Ludwiczuk (2017)[56] and Zidorn (2021)[53], the presence of flavonoids in all individual samples evaluated may be due to the fact that these compounds belong to the group of SM with the greatest distribution in nature and because they are the plant's first line of defense against various pollutants, plagues, and other plants. Moreover, consulted references have demonstrated abundance incidence of xanthones, flavones, and flavonols in *Croton rivinifolius*[20]. While compounds such as alkaloids are restricted to some botanical families (only are found in 20% of species of vascular plants)[57]. However, there is a study where *C. elegans* leaves collected at the parish of Sagrario in Cotocachi were extracted with hexane and its residual plant was submitted to acidic extraction in order to obtain the alkaloid fraction (two morphinan alkaloids. (+)-pallidine and (+)-methylpallidine)[58]. Another investigation has reported the principal alkaloidal crotsparinine, crotsparine and sparsiflorin, extracted through bioassay-guided fractionation of *Croton sparsiflorus* Morong[59]. So, no detection of alkaloids in this work could be due to the solvent used in the extraction technique used.

4.3. Total phenolic content and total flavonoid content

Phenolic compounds in plants are known to act as free radical scavengers and the antioxidant activity of most of the plant produce is mainly due to the presence of this compounds[55,60,61]. Basically, antioxidant mechanism of polyphenolic compounds is based on their hydrogen donating and metal ion chelating abilities[55,60]. Flavonoids are the most common and widely distributed important single group of phenolics present in plants that are highly effective as antioxidants[62]. As mentioned above, the adaptation and defense of the plant to UV-B radiation is the increased production of secondary metabolites as phenols and flavonoids, which absorb radiation in the UV-B range and have radical scavenging activity[55].

In high-altitude regions, plants are challenged by unfavorable or even adverse abiotic environmental conditions that affect growth dynamics and threaten their existence [18]. In this context, the altitude gradient is associated with a wide variation in environmental conditions, which affect the metabolic expression. Despite this, the results obtained in the present work (Table 5) indicated that, the total content of phenolic compounds and flavonoids in leaves of *C. elegans* is not correlated to elevation and neighborhood index. This study topic has not been evaluated in the *C. elegans* species, however, reports in flowering heads collected from different altitudes in *Crepis capillaris* (180 – 1060 masl), *Hieracium pilosella* (190 – 1290 masl) and *Hypochaeris radicata* (20 – 1290 masl) showed a positive correlation between the altitude of the growing site and the contents of flavonoids and phenolic acids within the flowering heads[63]. In another research, leaves of *Nepeta septemcrenata*, *Origanum syriacum* subsp. *Sinaicum*, *Phlomis aurea*, *Rosa arabica*, and *Silene schimperiana* which were collected from different site between 1600 and 2200 meters above sea level shown a progressive increase of total phenols and flavonoids in response to different altitudes[64]. Our results indicate that an altitudinal gradient range 2241 – 2366 m does not present substantial differences in ecological factors, or higher abiotic stress at the higher altitude of this study, which could cause a significant difference expression of total phenol and total flavonoid content.

In general, high values were observed in the phenolic and flavonoid content of the extracts of the evaluated samples (> 140 mg GAE/g DWE and > 170 mg QE/g DWE, respectively), which were higher than those found in previous studies for species of this genus. *C. elegans* is used in the traditional medicine of Ecuador to treat fever[8] and these applications could be related to the high proportion of phenolic compounds found.

5. CONCLUSION

This is the first report that evaluates the effect of elevation zone and neighborhood index in the metabolic expression of leaf methanolic extracts of *Croton elegans*. The results of qualitative phytochemical screening revealed the presence of the same primary metabolites (carbohydrates) and secondary metabolites analyzed (flavonoids, phenolic compound, and glycosides) in all extracts of *Croton elegans*. The highest amount of phenolic and flavonoid content in extracts *of C. elegans* leaves was found in TL-2 sample with 155.22 mg GAE/g DWE and 329.77 mg QE/g DWE, respectively. On the other hand, the elevation gradient in range 2241 – 2366 masl and neighborhood index did not present a statistically significant difference in the percentage of yield, production of primary and secondary metabolites and in the total content of phenols and flavonoids.

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7. FIGURES

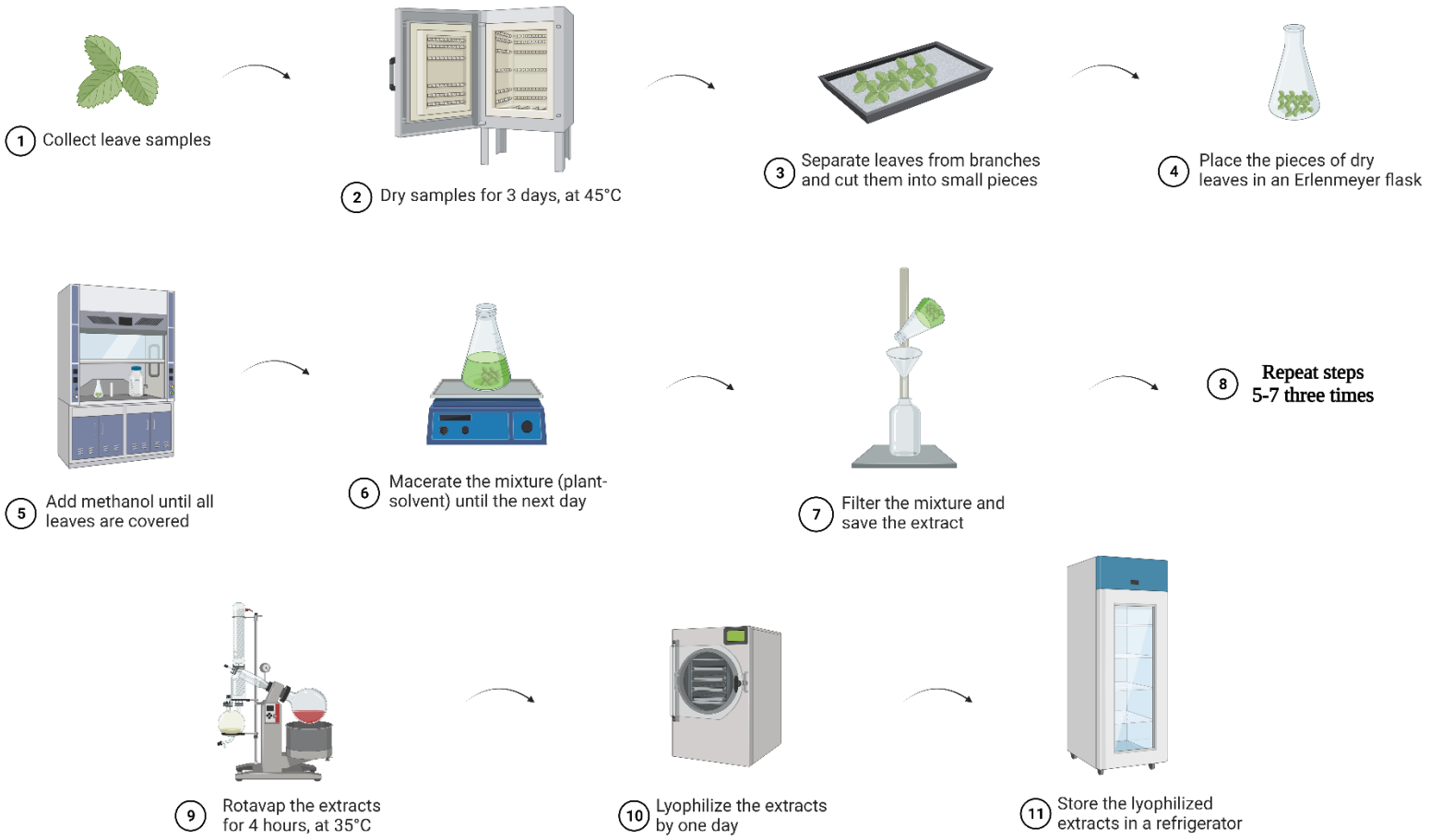

Figure 1. Schematic illustration of methanolic extraction protocol used. (Created with BioRender.com)

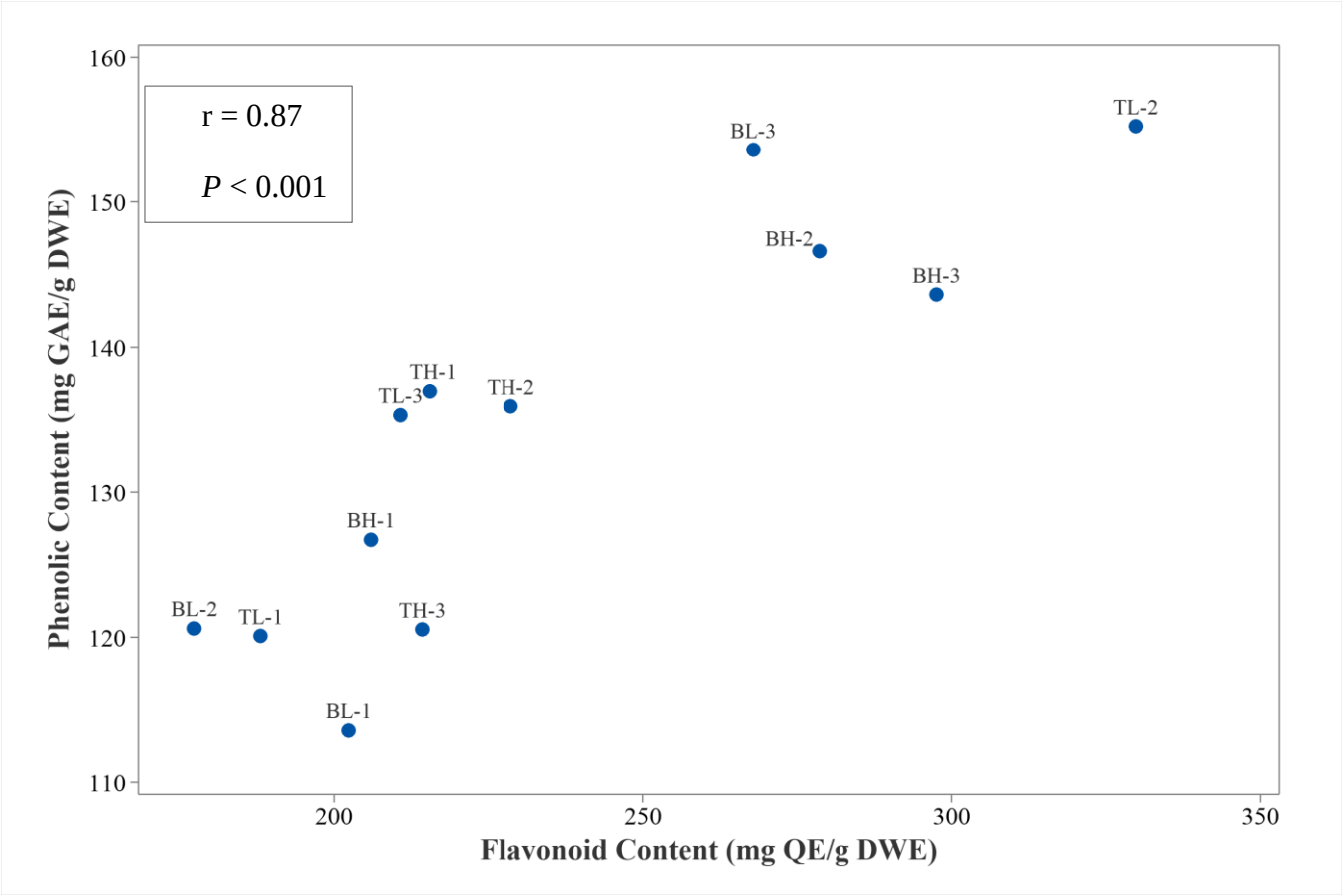

Figure 2. Relation between total phenolic content (mg GAE/g DWE) and total flavonoid content (mg QE/g DWE) in leaves of 12 *Croton elegans* individuals sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. A strong Pearson correlation was detected between the two variables.

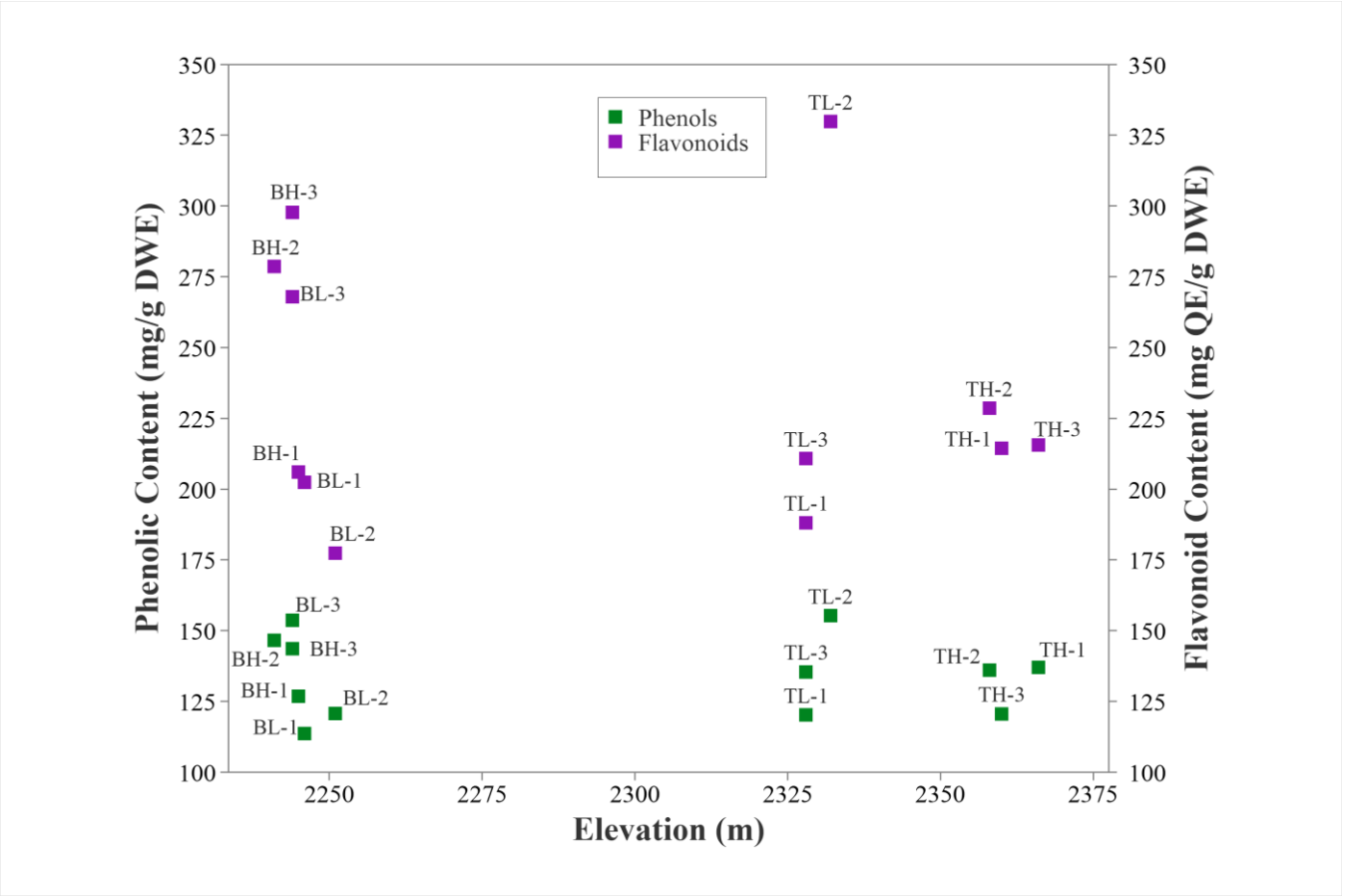

Figure 3. Variation, along the elevation gradient, of total phenolic content (mg GAE/g DWE) and total flavonoid content (mg QE/g DWE) in leaves of 12 *Croton elegans* individuals sampled at San Eloy Hill, Yachay Botanical Garden, Urcuquí. Ecuador.

8. TABLES

Table 1. Sampling design for the study.

		Location in the hill	
		Hilltop	**Hill base**
Neighborhood index	**Low**	3 individuals	3 individuals
	High	3 individuals	3 individuals

Table 2. Geographic characteristics and habitat description of *Croton elegans* sampling sites at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador. Plant samples were obtained in February 2022. Sample codes mean: T= Hilltop, B= Hill base, H= High neighborhood index, L= Low neighborhood index.

Sample code	Latitude	Longitude	Elevation (m)	General habitat description
TH-1	0.4178	-78.18534	2366	Hilltop with relatively high density of different plant species.
TH-2	0.41785	-78.18561	2358	
TH-3	0.41722	-78.18439	2360	
TL-1	0.41868	-78.18423	2328	Hilltop with relatively low neighborhood of different plant species.
TL-2	0.41843	-78.18431	2332	
TL-3	0.41868	-78.18423	2328	
BH-1	0.41487	-78.18486	2245	Hill base with relatively high density of different plant species.
BH-2	0.41928	-78.18182	2241	
BH-3	0.41475	-78.18489	2244	
BL-1	0.41492	-78.18485	2251	Hill base with relatively low neighborhood of different plant species.
BL-2	0.41492	-78.18488	2246	
BL-3	0.41928	-78.18182	2241	

Table 3. Methanolic extract yields from leaves of different *Croton elegans* individuals sampled at different sites at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador.

Sample Code [1]	Dry Weight (g)	Extract (g)	Yield (%)
TH-1	10.06	1.7637	17.5
TH-2	10.01	1.8714	18.7
TH-3	10.00	1.7745	17.7
TL-1	10.34	1.7234	16.7
TL-2	10.21	2.0322	19.9
TL-3	10.14	2.0054	11.7
BH-1	10.14	1.3984	13.8
BH-2	10.2	1.5536	15.2
BH-3	10.15	1.8542	18.3
BL-1	10.04	1.683	16.8
BL-2	9.96	1.5555	15.6
BL-3	10.02	1.4828	14.8

[1] Sample codes are as in **Table 2**: T= Hilltop, B= Hill base, H= High neighborhood index, L= Low neighborhood index.

Table 4. Qualitative phytochemical screening of *Croton elegans* methanolic extracts from plant samples collected at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador.

Secondary metabolites	Chemical test	Sample code [1]											
		TH-1	**TH-2**	**TH-3**	**TL-1**	**TL-2**	**TL-3**	**BL-1**	**BL-2**	**BL-3**	**BH-1**	**BH-2**	**BH-3**
Alkaloids	Wagner	-	-	-	-	-	-	-	-	-	-	-	-
	Mayer	-	-	-	-	-	-	-	-	-	-	-	-
	Dragendroff	-	-	-	-	-	-	-	-	-	-	-	-
	Iodine	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	Alkaline reagent	+	+	+	/	/	/	+	+	+	+	+	+
	Shinoda	+	+	+	+	+	+	+	+	+	+	+	+
	Concentrate H_2SO_4	+	+	+	+	+	+	+	+	+	+	+	+
Phenolic compounds	Iodine	+	+	+	+	+	+	+	+	+	+	+	+
	Ferric chloride	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	Braymer	-	-	-	/	/	/	-	-	-	-	-	-
	10% NaOH	-	-	-	-	-	-	-	-	-	-	-	-
Saponins	Foam test 1	-	-	-	-	-	-	-	-	-	-	-	-
	Foam test 2	-	-	-	-	-	-	-	-	-	-	-	-
	$NaHCO_3$	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates	Molish	+	+	+	+	+	+	+	+	+	+	+	+
	Resorcinol	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	Borntrager	-	-	-	-	-	-	-	-	-	-	-	-
	Aqueous NaOH	-	-	-	-	-	-	-	-	-	-	-	-
	Concentrate H_2SO_4	+	+	+	+	+	+	+	+	+	+	+	+
Cardiac Glycosides	Keller-Killani	+	+	+	+	+	+	+	+	+	+	+	+
Proteins and Amino acids	Ninhydrin	-	-	-	-	-	-	-	-	-	-	-	-

Each sample was run in triplicates. (+) indicates the presence of the secondary metabolite. (-) indicates the absence of the secondary metabolite. (/) indicates the possible presence of the secondary metabolite. [1] Sample codes are as in **Table 2**: T= Hilltop, B= Hill base, H= High neighborhood index, L= Low neighborhood index.

Table 5. Total phenolic and flavonoid content of *Croton elegans* methanolic extracts from plant samples collected at San Eloy Hill, Yachay Botanical Garden, Urcuquí, Ecuador.

Sample Code [1]	mg GAE/g DWE [2]	mg QE/g DWE [3]
TH-1	136.99 ± 0.93	215.49 ± 17.98
TH-2	135.94 ± 0.59	228.58 ± 16.10
TH-3	120.54 ± 2.85	214.30 ± 17.98
TL-1	120.09 ± 0.59	188.11 ± 14.29
TL-2	155.22 ± 0.26	329.77 ± 14.43
TL-3	135.35 ± 4.53	210.73 ± 7.43
BH-1	126.72 ± 1.23	205.97 ± 15.57
BH-2	146.59± 0.34	278.58 ± 11.48
BH-3	143.61 ± 3.16	297.63 ± 27.28
BL-1	113.62 ± 1.98	202.39 ± 21.72
BL-2	120.61 ± 3.18	177.39 ± 18.90
BL-3	153.58 ± 3.87	267.87 ± 26.81

[1] Sample codes are as in **Table 2**: T= Hilltop, B= Hill base, H= High neighborhood index, L= Low neighborhood index.
[2] Units for total phenolic content are expressed in mg of gallic acid equivalent (GAE) per g of dry weight extract (DWE). [3] Units for total flavonoid content are expressed in mg of quercetin equivalent (QE) per g of dry weight extract (DWE).
Values represent the mean of triplicate measurements ± standard deviation.

9. SUPPLEMENTARY MATERIAL

Table S1. Field photos of the individuals studied.

Code sample	Field photo	Height (cm)	Cover (cm^2)
TH-1		60	5894
TH-2		54	8738
TH-3		41	8877
TL-1		50	8380
TL-2		33	7678
TL-3		48	5596

BH-1		63	18118
BH-2		60	15174
BH-3		45	5268
BL-1		42	9702
BL-2		42	5404
BL-3		73	8646