

Lithium biofortification of Brassicaceae baby leaves by soilless cultivation: nutritional value and implications on the *Drosophila melanogaster*

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ABSTRACT: We accessed the impact of root application of Li carbonate (Li_2CO_3) on the productivity, nutritional, and bioactive properties of species of the Brassicaceae family. In addition, the species and Li concentrations that exhibited the highest content of bioactive compounds and agronomic characteristics were investigated regarding their impact climbing ability of the *Drosophila melanogaster*. The species of the Brassicaceae family (mustard (*Brassica juncea*), purple cabbage (*Brassica oleracea* var. capitata f. rubra), arugula (*Eruca vesicaria* ssp. *sativa*), and kale (*Brassica oleracea* L. var. *acephala* D.C.)) were cultivated in a nutrient solution enriched with Li_2CO_3 (0, 15, 30, 60, and 120 mg.L^{-1}) for 15 days. Purple cabbage has the highest lithium absorption capacity (860.55 ppm), followed by arugula (722.43 ppm), kale (688.77 ppm), and mustard (485.48 ppm). Lithium accumulation demonstrated a dose-dependent effect, with Li_2CO_3 at 60 mg.L^{-1} showing the most significant growth effect on arugula compared to other tested doses. Arugula was the most promising species in the study, and at a concentration of 60 mg.L^{-1} , it had the highest phenolic content ($584.9 \pm 8.08 \mu\text{mol/g}$) and antioxidant activity (DPPH: $11.063 \pm 170.2 \mu\text{mol/g}$; ABTS: $24.481 \pm 1.950 \mu\text{mol/g}$). It was also the second species with the best agronomic performance, after mustard. The arugula extracts were found to positively impact the survival rate and climbing ability of the *Drosophila melanogaster*. The results demonstrated that it is feasible to enhance the antioxidant activity of brassicas by supplementing them with lithium. Furthermore, extracts of brassicas exhibited favorable outcomes in tests with *D. melanogaster*, which might contribute to enhanced neuromuscular effects.

Key words: arugula, Li_2CO_3 , bioactive compounds, hydroponic, *Drosophila melanogaster*.

Biofortificação com lítio de folhas de Brassicaceae por cultivo sem solo: valor nutricional e implicações em *Drosophila melanogaster*

RESUMO: O objetivo deste estudo foi avaliar o impacto da aplicação radicular de carbonato de lítio (Li_2CO_3) na produtividade, propriedades nutricionais e bioativas de espécies da família Brassicaceae. Além disso, foram investigadas as espécies e concentrações de Li que apresentaram maior conteúdo de compostos bioativos e características agrônomicas quanto ao impacto na habilidade de escalada de *Drosophila melanogaster*. As espécies da família Brassicaceae (mostarda (*Brassica juncea*), repolho roxo (*Brassica oleracea* var. capitata f. rubra), rúcula (*Eruca vesicaria* ssp. *sativa*) e couve (*Brassica oleracea* L. var. *acephala* D.C.)) foram cultivadas em solução nutritiva enriquecida com Li_2CO_3 (0, 15, 30, 60 e 120 mg.L^{-1}) por 15 dias. O repolho roxo tem a maior capacidade de absorção de lítio (860,55 ppm), seguido pela rúcula (722,43 ppm), couve (688,77 ppm) e mostarda (485,48 ppm). O acúmulo de lítio demonstrou um efeito dose-dependente, com Li_2CO_3 a 60 mg.L^{-1} apresentando o maior efeito de crescimento na rúcula em comparação com as outras doses testadas. A rúcula foi a espécie mais promissora do estudo e, em uma concentração de 60 mg.L^{-1} , apresentou o maior conteúdo fenólico ($584,9 \pm 8,08 \mu\text{mol/g}$) e atividade antioxidante (DPPH: $11.063 \pm 170,2 \mu\text{mol/g}$; ABTS: $24.481 \pm 1.950 \mu\text{mol/g}$). Foi também a segunda espécie com o melhor desempenho agrônômico, depois da mostarda. Os extratos de rúcula mostraram impacto positivo na taxa de sobrevivência e na habilidade de escalada de *Drosophila melanogaster*. Os resultados demonstraram que é viável aumentar a atividade antioxidante de brassicas por meio da suplementação com lítio. Além disso, os extratos de brassicas exibiram resultados favoráveis em testes com *D. melanogaster*, o que pode contribuir para efeitos positivos na função neuromuscular.

Palavras-chave: rúcula, Li_2CO_3 , compostos bioativos, hidroponia, *Drosophila melanogaster*.

INTRODUCTION

Biofortification involves the fortification of crops with essential minerals and nutrients, thereby enhancing their nutritional value. As a result, the population's consumption of minerals has been on the rise. This can positively impact public health by

reducing nutrient deficiencies and promoting better nutrition (GUPTA et al., 2021). Lithium, traditionally considered a non-essential mineral for plant metabolism, has gained attention due to its beneficial effects as a mood stabilizer and its widespread use in psychiatry for conditions such as bipolar disorders, anxiety, autism, depression, and neurodegenerative

diseases such as Alzheimer's disease (SCHÄFER, 2012). Food sources of Li include vegetables, grains, and yeasts, with varying amounts ranging from 0.2 to 30 mg. kg⁻¹. Li can occur naturally in soil or because of contamination, leading to different element levels in crops grown in environments where the soil is enriched with Li, depending on its concentration (SHAHZAD et al., 2016). Consequently, the application of Li in vegetables appears as a relatively unexplored area of study. Current research has primarily focused on leafy vegetables such as lettuce and a traditional Chinese herb called *Apocynum venetum*. However, there is still potential for further investigation into the effects of Li supplementation in a broader variety of vegetables (JIANG et al., 2018; SILVA et al., 2019).

Despite the lack of conclusive evidence regarding the application of Li in plants, several studies have highlighted the significant effects of a deficiency of this trace element in the human body. Adequate levels of Li in the human body require regular consumption of foods or derivatives containing enough of this element, as Li does not accumulate and only exerts a regulatory effect. This highlighted the importance and necessity of research involving the biofortification of foods with Li (NAEEM et al., 2021; RYBAKOWSKI et al., 2018).

Brassicaceae plants offer a promising possibility for the efficient delivery of sufficient doses of Li, and it is very popular, relatively inexpensive, easy to germinate and grow, and have great potential health-benefits thanks to their high content of glucosinolates, vitamins, and polyphenols (RAO et al., 2020). Thus, Brassicaceae plants are suitable candidates for biofortification with Li, allowing for the evaluation of their mineral absorption capacity. However, few studies demonstrated these characteristics concerning Li absorption in Brassicaceae plants (DI GIOIA et al., 2019). Therefore, this study was to assess the impact of root application of Li carbonate (Li₂CO₃) on the productivity, nutritional, and bioactive properties of species of the Brassicaceae family. In addition, the species and Li concentrations that exhibited the highest content of bioactive compounds and agronomic characteristics were investigated regarding their potential neuromuscular effect in *Drosophila melanogaster*.

MATERIALS AND METHODS

Plant material, growth conditions and sample harvest and storage

The plant material utilized in the research was cultivated in June 2022 using the nutrient film

technique (NFT) hydroponic system installed in an agricultural greenhouse situated in Cuiabá, Mato Grosso, Brazil (latitude 15°36'41.6"S and longitude 56°03'54.2"W, at an altitude of 176 meters). The agricultural greenhouse had a height of 3.5 meters and was equipped with a PAD&FAN evaporative cooling system. The activation and deactivation temperatures for the cooling system were set at 28 °C and 24 °C, respectively. The temperature of the environment ranged between 22 °C and 32 °C. The sides and roof of the protected environment were covered with a 150-micron plastic film, providing 60% diffusion of photosynthetically active radiation (PAR) and 85% transmissivity of PAR light (Suncover defuse, Ginegar Polysack®).

The four cultivated species were mustard (*Brassica juncea*), purple cabbage (*Brassica oleracea* var. capitata f. rubra), arugula (*Eruca vesicaria* ssp. *sativa*), and kale (*Brassica oleracea* L. var. *acephala* D.C.), all belonging to the Brassicaceae family and obtained from Isla Sementes®. The cultivation process was divided into three stages. The first stage involved sowing the crops in polyethylene trays with 200 cells filled with commercial Carolina Soil® substrate. Between 10 and 15 seeds were sown per cell of the trays. Subsequently, the seeds were covered with a thin substrate layer and kept in a dark environment for 48 hours. During this period, light sprinkler irrigation was carried out every 12 hours. In second stage, the trays were placed on a germination table with a sub-irrigation system automatically activated for 5 minutes every hour during the daytime. The same nutrient solution was used in the first, second and third phases. The nutrient solution was adjusted daily according to pH (5.5 to 6.5) and electrical conductivity (1.0 mS cm⁻¹). This stage lasted for 13 days, resulting in 15 days for the seedling production period. The nutrient solutions used in all treatments were prepared with the following quantities of fertilizers (excluding Li carbonate): 660 mg. L⁻¹ of Hidrogood Fert (N:P:K:Mg:S, 9:9:29:3:4), 45 mg. L⁻¹ of iron chelate, and 495 mg. L⁻¹ of calcium nitrate. The electrical conductivity (HM Digital, COM-80 HM, USA) and pH (HM Digital, PH-80 HM, USA) were monitored using a portable digital conductivity meter and pH meter.

In the third stage, the seedlings of the four species were transferred to an NFT hydroponic system. The system consisted of 20 hydroponic benches, each containing 6 channels with a width of 8 cm and a length of 100 cm (R80 Profile, Hidrogood®). Each mini-bench had 30 planting holes and served as an experimental unit. The plants were spaced 12.5 cm

apart within a row, and there was also a 12.5 cm spacing between rows on the bench. The supply of nutrient solution to plants was done automatically and intermittently. During the daytime, the system alternated every 15 minutes, while during the nighttime, it was activated for 5 minutes with a 2-hour interval. The nutrient solutions were prepared at the beginning of this stage in 100-liter reservoirs, one for each treatment (Li doses). Daily measurements and adjustments of the nutrient solution were performed, maintaining the pH of 5.5 to 6.5 and electrical conductivity at 1.5 mS.cm^{-1} ($\pm 0.05 \text{ mS.cm}^{-1}$). And once every 7 days the containers were cleaned to change the entire solution and readjust the dose of lithium applied. This stage lasted for 15 days, resulting in 30 days of cultivation. A period of 30 days was stipulated as an average time for the growth of the four cultivated species (BANTIS et al., 2021), with all procedures executed on the same day to minimize plant differences. The fresh weight of each plant was measured using a precision balance (OHAUS, adventurer ARD110, USA). Plant height was measured using a millimeter ruler (cm), and the count of plants and leaves was recorded, considering the species and the respective Li concentration applied.

Li biofortification and experimental design

The experiment followed a completely randomized design, where lithium (Li) dosage was the treatment factor, with five different levels. Each hydroponic table represented a replication, totaling four replications per treatment. An identification code was assigned for each baby leaf variety, AR for arugula, KA for kale, MU for mustard, and PC for purple cabbage. The experiment analyzed the effects of lithium carbonate (Li) on nutrient solution reservoirs over a 15-day planting period. Five groups were tested: Group 0: Control, received only nutrient solution without Li carbonate; Group 15: Received 15 mg/L of Li carbonate; Group 30: Received 30 mg/L of Li carbonate; Group 60: Received 60 mg/L of Li carbonate; and Group 120: Received 120 mg/L of Li carbonate. Lithium was applied at the beginning of the third stage and in the middle of it after a complete change in the contents of the reservoir. All the species were subjected equally to all the application groups, and the presentation format of the species subjected to different treatments was done according to the following examples: AR-0 (arugula, 0 mg.L^{-1}), AR-15 (arugula, 15 mg.L^{-1}), AR-30 (arugula, 30 mg.L^{-1}), AR-60 (arugula, 60 mg.L^{-1}), and AR-120 (arugula, 120 mg.L^{-1}).

Mineral profile

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was utilized for mineral quantification, including Li and other target minerals. The microwave digestion process involved adding 100 mg of the sample, 2.5 mL of distilled sub-boiling 20% (v/v) nitric acid (HNO_3), and 1.5 mL of 30% (v/v) hydrogen peroxide (H_2O_2) to specific Teflon tubes. The tubes were then subjected to a microwave digestion process (25 min, 850W); after digesting and cooling the Teflon tubes, the digestate volume was adjusted to 10 mL with ultrapure water, and the analysis was performed using ICP-OES (Thermo Scientific, iCAP 7000 Series). The concentrations of Li, macro- and micronutrients in plant samples were determined through inductively coupled plasma optical emission spectrometry (ICP-OES) and are reported in mg kg^{-1} .

Determination of total phenolic compounds

The analytical extracts to analyze total phenolic compounds and antioxidant activity were prepared following the procedures described by (BACHIEGA et al., 2016). The quantification of phenolic compounds was performed in triplicate using the Folin-Ciocalteu reagent method following the protocol described by (WOISKY & SALATINO, 1998). Total phenolic compounds (TPC) were quantified by constructing a standard curve using gallic acid and the results were expressed in mg of gallic acid equivalents (GAE) per gram of the sample.

Total antioxidant activity (TAA)

This study employed three chemical methods to determine the antioxidant activity of the extracts under investigation. These methods included: (1) Scavenging capacity of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) described by BRAND-WILLIAMS et al. (1995); (2) Scavenging capacity of the free radical 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic (ABTS) according the method proposed by NENADIS et al. (2004); (3) Ferric reducing antioxidant power (FRAP) according the methodology proposed by PULIDO et al. (2000).

In vivo (Drosophila melanogaster) experimental trial

All animals utilized in this project (wild-type strain: Canton Special) were maintained in vials or sealed plastic bottles with cotton or gauze (material that permits aeration) within a controlled environment at temperature of $25 \pm 1^\circ\text{C}$ and a photoperiod

(light/dark) of 12/12 hours. The standard diet for *Drosophila melanogaster* consisted of an enriched medium containing corn flour (6.5%), yeast extract (6.5%), agar (3%), and methyl-p-hydroxybenzoate (3%) (DE ALENCAR et al., 2023). Aqueous extracts (1:10 (w/v)) AR 0, AR 60, and AR 120 were used to evaluate the effect of lithium on the animal's diet. Every two days, the animals were transferred to vials containing fresh food to maintain optimal physiological conditions and prevent the death of *D. melanogaster* due to other causes, such as sticking to wet food, mold growth, or bacterial development (BOSCO et al., 2015).

To ascertain the survival rates of the insects, each tube containing the different feeding conditions and *D. melanogaster* was monitored for a period of 30 days. The number of living and dead insects was recorded. In addition, the climbing activity of the insects was measured as described by (BENZER, 1967), using a counterflow experiment conducted over 30 days. The results were obtained using the phototaxis index (ZIEGLER et al., 2016).

Statistical analysis

Statistical analysis was performed using ANOVA, followed by Tukey's test, Pearson correlation and analysis of principal component using the GraphPad Prism version 8.0 and XLSTAT software. Results were considered significant at ($P < 0.05$). The analysis was performed separately for each species, where the variables were observed between the different concentrations. The data are presented as mean $\pm$ SEM for the analysis of flies.

RESULTS

Agronomic data

The results of agronomic data are expressed as an average (Table 1). The measures evaluated respond significantly to the addition of lithium; although, the species was the variable that most influenced the agronomic data (Table 2).

The average height in KA-0 was (27.35 ± 5.00 cm), significantly lower than (28.98 ± 3.14 cm) in KA-60, indicating a 5.6% increase ($P <$

Table 1 - Average agronomic data for the four varieties of biofortified brassicas with different levels of Li.

Treatment (mg.L ⁻¹ of Li ₂ CO ₃)	Height (cm)	Number of leaves**	Weight (g)
-----Kale-----			
0	27.35 ± 5.00^b	65.69 ± 16.71^a	32.51 ± 13.30^a
15	27.55 ± 3.67^a	59.94 ± 12.71^b	23.38 ± 6.10^b
30	25.73 ± 2.89^b	61.81 ± 14.87^b	29.31 ± 8.80^a
60	28.98 ± 3.14^a	66.06 ± 16.80^a	26.60 ± 9.50^a
120	20.75 ± 4.90^c	45.00 ± 17.58^c	8.97 ± 3.40^c
-----Arugula-----			
0	26.04 ± 2.13^a	72.31 ± 18.51^a	37.15 ± 8.48^a
15	25.18 ± 2.23^a	74.69 ± 19.30^a	30.81 ± 9.26^a
30	23.37 ± 2.76^b	62.31 ± 12.08^b	25.64 ± 6.32^b
60	25.73 ± 2.96^a	62.50 ± 18.02^b	34.77 ± 11.39^a
120	20.98 ± 2.15^b	53.81 ± 12.08^c	20.79 ± 6.31^b
-----Mustard-----			
0	34.25 ± 4.29^b	94.31 ± 19.10^a	98.35 ± 21.50^a
15	35.06 ± 3.20^a	93.44 ± 21.78^a	88.29 ± 22.11^b
30	37.31 ± 3.95^a	96.06 ± 18.06^a	115.26 ± 25.54^a
60	34.38 ± 4.28^b	82.00 ± 16.60^b	89.40 ± 26.92^b
120	29.74 ± 3.48^c	77.44 ± 13.69^b	68.49 ± 14.02^c
-----Purple cabbage-----			
0	22.90 ± 3.80^a	56.13 ± 19.00^a	16.96 ± 7.87^a
15	20.09 ± 3.51^a	52.13 ± 14.96^a	10.98 ± 5.09^a
30	17.84 ± 3.15^b	40.13 ± 18.00^a	8.84 ± 5.80^b
60	22.00 ± 3.56^a	45.00 ± 16.14^a	12.28 ± 5.20^a
120	18.78 ± 4.14^b	44.63 ± 19.79^a	9.55 ± 6.15^b

*Different letters in the column indicate that the values differ significantly according to Tukey's test ($P < 0.05$); ** leaves per experimental unit which contained around 10 plants.

Table 2 - ANOVA data for agronomic data, phenolic compounds, antioxidant activity and mineral content.

	Height (cm)	N of leaves	Weight (g)	TPC	DPPH	FRAP	Li	B	Mg	P	S	K	Ca	Mn	Fe	Cu	Zn	ABTS
R ²	0.91	0.84	0.96	0.99	0.99	0.91	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.90
F	32	16	79	441	310	33	71233	1337	4903	25165	31771	2107	5514	16865	18392	2581	21283	27
Pr > F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Specie F	168	82	458	2104	1593	13.03	18606	1379	21391	13023	17433	2112	25607	82774	38950	9407	60760	72
Pr > F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
% Li F	18	9.47	16	135	18	58	31197	1321	3663	5618	10810	5446	2581	10487	38980	1500	36948	8.36
Pr > F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Specie*%Li F	3.10	1.52	5.23	127	86	29	4143	1332	1194	5414	3118	993	1468	2513	6389	1235	6193	22
Pr > F	0.002	0.143	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

0.05). However, in KA-120, the average height was significantly lower (20.75 ± 4.90 cm), representing a notable decrease of 24.1% compared to KA-0 ($P < 0.05$). Regarding the average number of kale leaves, the KA-0 had (65.69 leaves per experimental unit), while the highest result was observed in KA-60 (66.06 leaves per plant), indicating a 1.51% increase compared to KA-0 ($P < 0.05$). The lowest result was obtained in KA-120 (45 leaves per experimental unit), which was 31.4% lower than the KA-0 ($P < 0.05$). In terms of average weight, KA-0 showed a high result (32.51 ± 13.3 g), while the KA-120 had the lowest result (8.97 ± 3.4 g), which was 72.4% lower than KA-0 ($P < 0.05$). These findings indicated that; although, the kale species showed interesting results in KA-60 (Table 1).

In arugula, AR-0 exhibited the highest average plant height of (26.04 ± 2.13 cm) ($P < 0.05$). Conversely, the lowest average plant height was observed in AR-120, measuring (20.98 cm), representing a 19.4% decrease compared to AR-0 ($P < 0.05$). As for the average number of leaves in arugula, AR-15 displayed the highest result with an average of (74.69 ± 19.30 leaves per experimental unit), indicating a 3.1% increase compared to AR-0. However, AR-120 showed the lowest result with an average of (53.81 ± 12.08 leaves per experimental unit), which was 25.5% lower than the AR-0 ($P < 0.05$). Regarding the average weight per arugula plant, no significant difference was observed among the AR-0, AR-15, and AR-60. Although, arugula plants showed different characteristics in other observed parameters, plant weight is a determining factor in productivity. In this case, AR-60 showed an improvement in weight (34.77 ± 11.39 g), which can be interpreted as positive in production, as it equals the weight of AR-0 without

statistical differences. The lowest result was also observed in AR-120 with an average weight of (20.79 ± 6.31 g), which was 44% lower than the AR-0 ($P < 0.05$). This indicated that biofortification with Li at the highest tested dose is not favorable for arugula in terms of fresh weight parameters.

Mustard plants presented 34.25 ± 4.29 cm of height in MU-0. The maximum plant height for mustard was observed in MU-30 with (37.31 ± 3.95 cm), representing an 8.2% increase compared to MU-0 ($P < 0.05$). The minimal plant height was found in MU-120, with (29.74 ± 3.48 cm), which was 19% lower than MU-0 ($P < 0.05$). Regarding the number of leaves per mustard plant, MU-0 had an average of 94.31 ± 19.10 leaves per experimental unit. The highest result was obtained in MU-30, representing a 1.82% increase compared to MU-0 (Table 1). The lowest result was observed in MU-120 with (77.44 ± 13.69 leaves per plant), which was 17.8% lower than MU-0 ($P < 0.05$). For the average weight per plant, mustard was obtained 98.35 ± 21.5 g in MU-0. The superior result was observed in MU-30 with 115.26 ± 25.54 g, representing a 14.6% improvement ($P < 0.05$). The lowest result was found in MU-120 with 68.49 ± 14.02 g, which was 30.3% lower than MU-0 ($P < 0.05$). This indicated that biofortification with Li is beneficial for mustard in terms of fresh weight in MU-30.

Purple cabbage had the highest average plant height in PC-0, with 22.90 ± 3.80 cm, and the lowest average plant height was found in PC-30, with 17.84 ± 3.1 cm, representing a 22% decrease compared to PC-0 ($P < 0.05$). Regarding the average number of leaves, PC-0 had an average of 56.13 ± 19 leaves per experimental unit, the highest result. The lowest result was obtained in PC-30 with 40.13 ± 18 leaves per experimental unit, representing a 28.5%

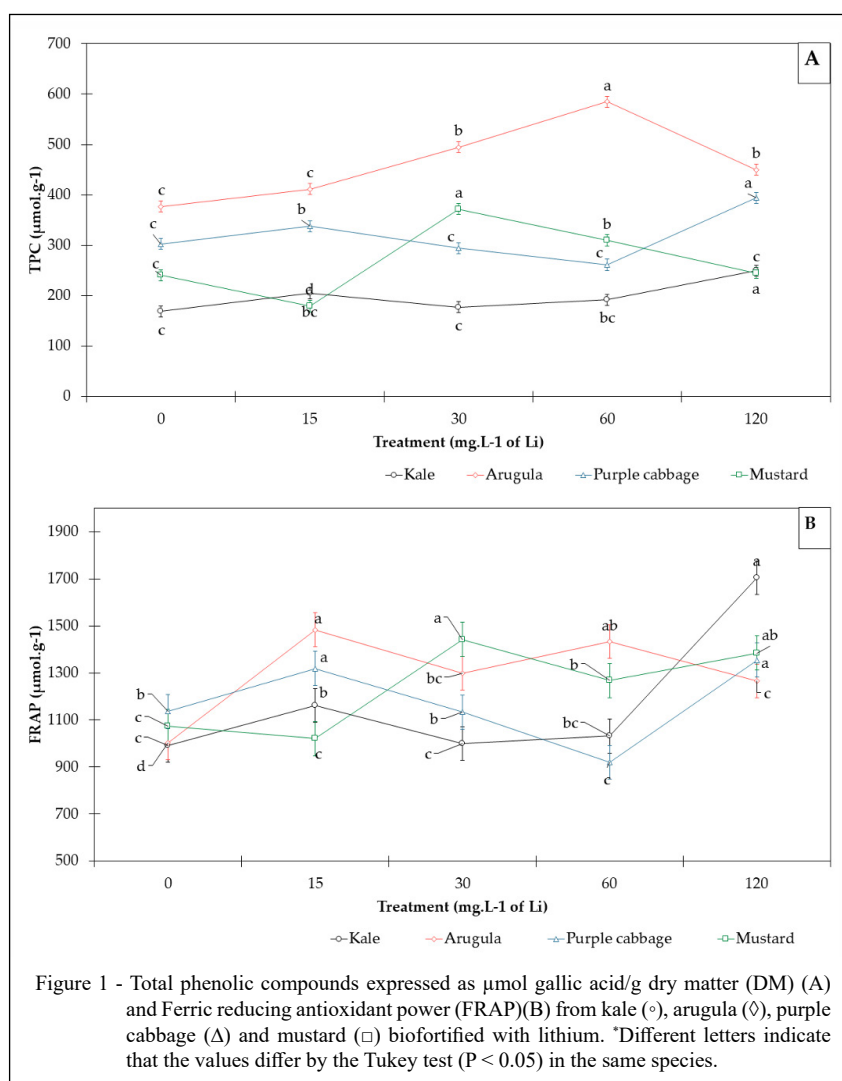
decrease compared to PC-0 ($P < 0.05$). In terms of weight per plant, purple cabbage obtained its highest result in PC-0 with 16.96 ± 7.87 g, and the lowest result was in PC-30 with 8.84 ± 5.80 g, which was 47.8% lower than PC-0 ($P < 0.05$). This indicated that biofortification with Li does not affect fresh weight at intermediate doses of purple cabbage.

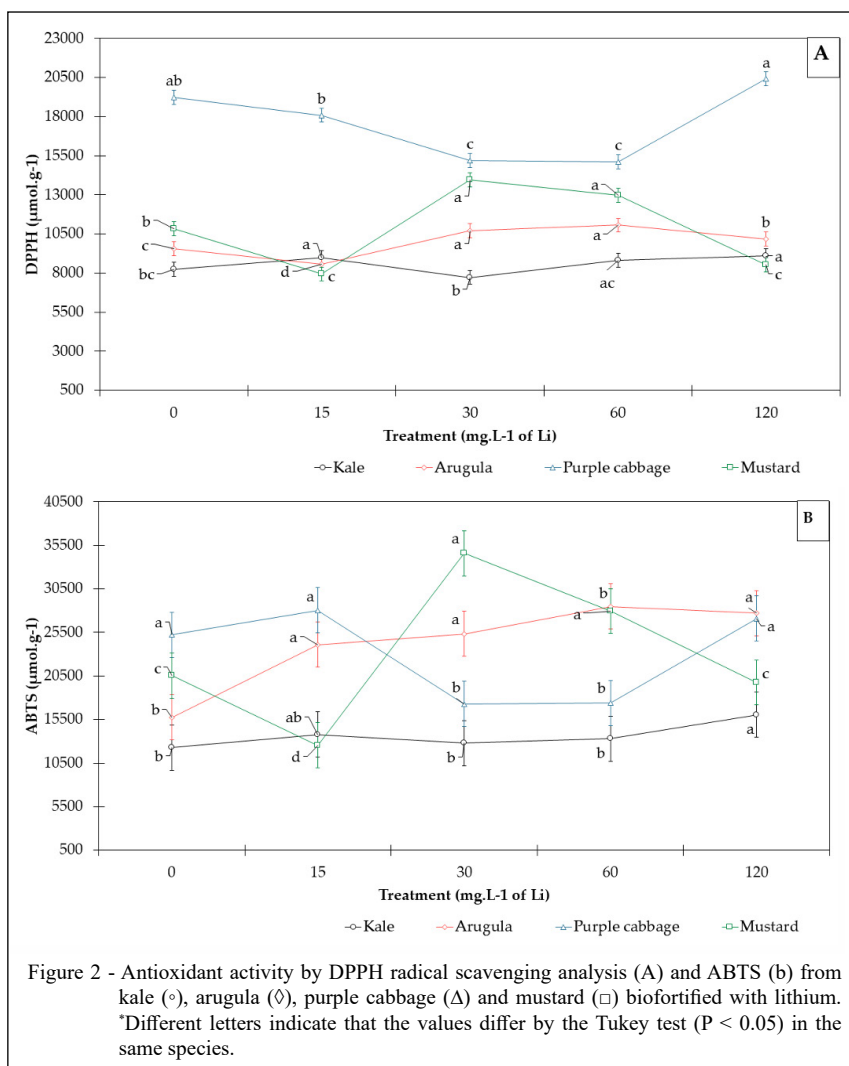
Total phenolic compounds and antioxidant activity

Biofortification with Li had a significant influence ($P < 0.0001$) on the number of phenolic compounds in the Brassicas species studied (Figure 1A, Table 2). The ANOVA analysis also showed that the species and the interaction* Li concentration were significant, although the species was the variable that most influenced the value of total phenolics (Figure 1A, Table 2). The same

behavior was observed in the antioxidant activity, where Li and species significantly influenced the antioxidant activity measured by DPPH, ABTS and FRAP (Figure 1B, Figure 2A and 2B, and Table 2).

Regarding the total phenolic compounds (TPC), statistical differences can be observed in kale, which had the lowest TPC content compared to other species (Figure 1A). KA-0 had the lowest TPC content with $(168.6 \pm 15.86 \mu\text{mol/g})$, while KA-120 showed the highest TPC content with $(249.6 \pm 5.10 \mu\text{mol/g})$, which was statistically lower ($P < 0.05$) than all other species. Arugula showed the highest levels of TPC among all groups and species studied, showing statistical differences in every treatment ($P < 0.05$) (Figure 1A). The group with highest TPC content was AR-60 ($584.9 \pm 8.08 \mu\text{mol/g}$), while AR-0 showed the lowest TPC content ($377.2 \pm 17.95 \mu\text{mol/g}$). This





indicated an increase in plant secondary metabolites due to stress caused by biofortification with Li. Purple cabbage presented statistical differences between all groups for TPC ($P < 0.05$), except between the PC-0 and PC-30 (Figure 1A).

Regarding antioxidant activity, measured by the FRAP method, the highest result was obtained in KA-120 with $(1,705 \pm 121 \mu\text{mol/g})$, which was statistically superior to other treatments ($P < 0.05$) (Figure 1B). In FRAP analysis of arugula, the superiority of AR-15 and AR-60 was observed, with $(1,483 \pm 50.26 \mu\text{mol/g})$ and $(1,433 \pm 109.1 \mu\text{mol/g})$, respectively, which were statistically different from the other groups of arugulas ($P < 0.05$) (Figure 1B). In the same way mustard showed superiority in MU-30 with $(1,443 \pm 69.50 \mu\text{mol/g})$, which was statistically higher than the other groups ($P < 0.05$) (Figure 1B).

For purple cabbage, the higher results were in PC-15 and PC-120, with $(1,319 \pm 58.04 \mu\text{mol/g})$ and $(1,355 \pm 24.21 \mu\text{mol/g})$, respectively, without statistical differences ($P < 0.05$). PC-60 was lower ($P < 0.05$) than other groups, with $(920.6 \pm 24.21 \mu\text{mol/g})$ (Figure 1B).

The DPPH radical scavenging analysis showed the highest values in purple cabbage, where PC-120 ($20,413 \pm 635.7 \mu\text{mol/g}$) was significantly higher ($P < 0.05$), followed by PC-0 ($19,230 \pm 1,033 \mu\text{mol/g}$) and PC-15 ($18,096 \pm 210.6 \mu\text{mol/g}$), with no statistical differences between them ($P > 0.05$) (Figure 2A). The lowest values were in PC-30 ($15,198 \pm 530.5 \mu\text{mol/g}$) and PC-60 ($15,115 \pm 602.9 \mu\text{mol/g}$), with no significant differences ($P > 0.05$). In kale, the highest values were in KA-15 ($8,972 \pm 115.2 \mu\text{mol/g}$) and KA-120 ($9,107 \pm 184.2 \mu\text{mol/g}$),

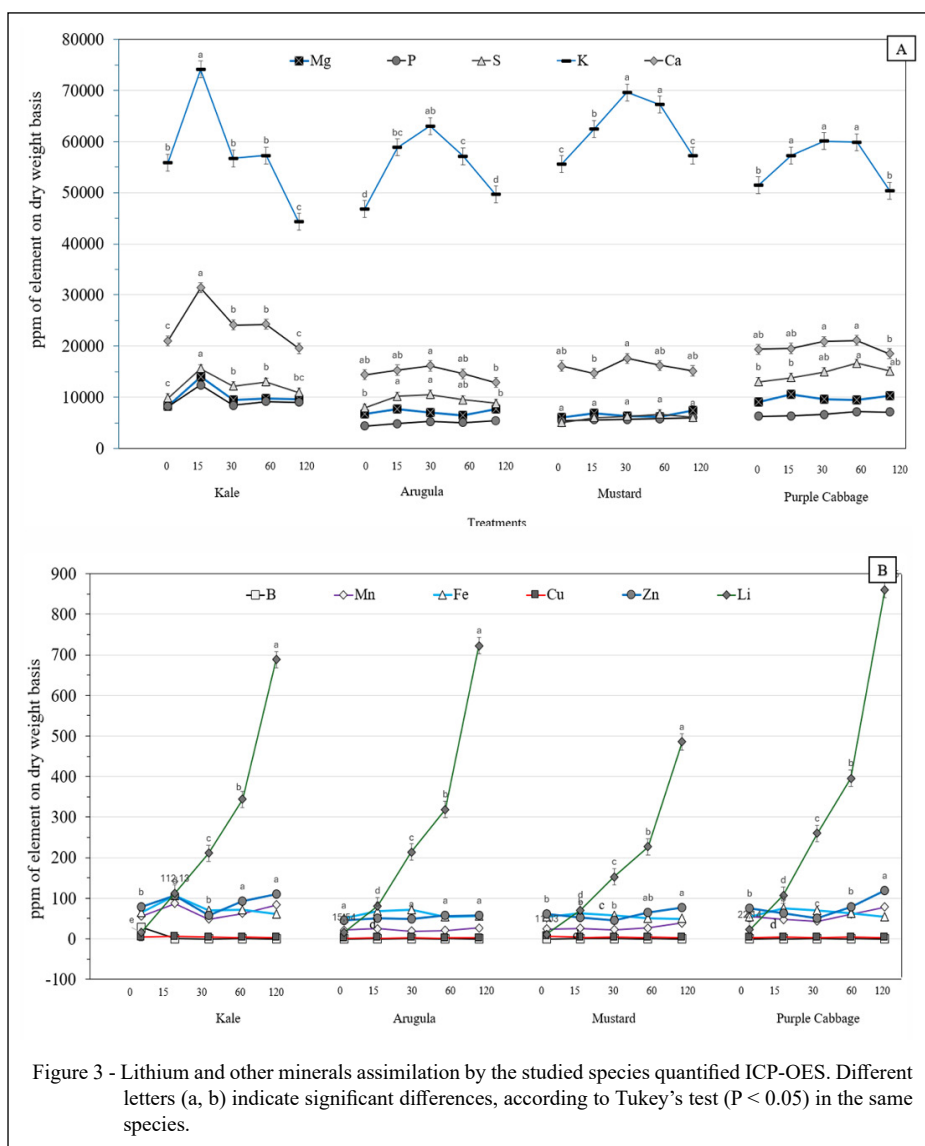
with no significant differences ($P > 0.05$). For DPPH analysis, MU-30 ($13,956 \pm 621.5 \mu\text{mol/g}$) and MU-60 ($12,972 \pm 283 \mu\text{mol/g}$) had the highest values, with no significant differences ($P < 0.05$) (Figure 2A). In arugula, antioxidant activity (DPPH and ABTS) was highest in AR-30, AR-60, and AR-120.

Finally, in ABTS analysis, the highest treatment was MU-30 with ($34,593 \pm 5,038 \mu\text{mol/g}$), which was statistically higher than the other treatments ($P < 0.05$). The worst result was observed in MU-15 with ($12,524 \pm 1,888 \mu\text{mol/g}$), which was statistically lower than all other treatments ($P < 0.05$) (Figure 2B). In kale, the highest result was observed in KA-120 ($16,052 \pm 683.1 \mu\text{mol/g}$), which was statistically different from all other groups of this species ($P <$

0.05), and the other groups did not show statistical differences ($P > 0.05$) (Figure 2B). Finally, in purple cabbage, PC-0, PC-15, and PC-120 displayed higher results compared to other groups without statistical differences between them ($P > 0.05$) (Figure 2B).

Li accumulation

The assimilation of Li by plants and other minerals is shown in figure 3. It can be observed that the absorption of Li by plants occurs in a dose-dependent manner in all analyzed species. In this sense, it is observed that the absorption and assimilation of Li were different in each species ($P < 0.001$) (Table 2), resulting in other optimal dosage points with Li about plant yield. This suggested that



each studied species had a specific behavior that could interfere positively or negatively depending on the dosage. Concerning Li absorption capacity among the species, the plants of each species were observed at a dose of 120mg.L^{-1} . Purple cabbage has the highest lithium absorption capacity with 860.55 ppm, followed by arugula, which absorbed 722.43 ppm in the same group, the following species in order of absorption is kale with 688.77 ppm, and finally, mustard was the species with the lowest lithium absorption capacity with 485.48 ppm.

Mineral profile

Figure 3A and 3B shows that biofortification with lithium does not change minerals linearly and that the difference varies according to mineral and the dose of biofortification with Li. However, the species and the biofortification with Li directly affected the concentration of Li in all species ($P < 0.001$). According to Pearson correlation the concentration of Li influenced negatively the concentration of B ($r = -0.233$, $p < 0.05$), K ($r = -0.393$, $P < 0.001$) and Fe ($r = -0.221$, $P < 0.05$); and positively the concentration of S ($r = 0.2256$, $P < 0.05$), Mn ($r = 0.3697$, $P < 0.05$) and Zn ($r = 0.5356$, $P < 0.001$).

When analyzing the content of macro minerals in kale, Ca, S, and K showed the same behavior with the increase in lithium content in plant, there was an increase in the content of these minerals at KA-15 (Ca: 31,387.88, S: 15,578.21, K: 74,109.72 ppm), with a decrease in values when compared to KA-120, where the amount of the same minerals was lower (Ca: 19,557.45, S: 10,959.12, K: 44,303.28 ppm) (Figure 3A). P and Mg also showed a similar behavior when compared, although present in smaller amounts among the macro minerals analyzed in species, they maintained a specific stability according to the doses of Li applied, with a slight increase in KA-15 with P: 12,396.93 Mg: 14,032.13 ppm, a higher amount when compared to KA-120 which presented P: 9,117.00 Mg: 9,566.05 ppm. The same behavior was repeated for the kale microminerals, which showed similar results in KA-0 (B: 27.95 Mn: 55.59 Fe: 64.40 Cu: 4.16 Zn: 78.48 ppm) and in KA-120 (B: 0.32 Mn: 83.01 Fe: 61.78 Cu: 3.13 Zn: 110.31 ppm).

When looking at the macro minerals in arugula, the behavior of K stands out from the others, being the only macro mineral with changes in its amount according to the dose offered reaching the highest amount in AR-30 (K: 63,052.97 ppm). Ca in AR-0 (Ca: 14,377.32 ppm), in AR-30 (Ca: 16,122.78 ppm), and in AR-120 (Ca: 12,871.68 ppm). The

other analyzed minerals showed a similar behavior, without significant changes with the increase in the supply of Li. For instance, AR-0 had the following concentrations: Mg: 6,728.61 P: 4,380.76 S: 7,892.68 B: 0.13 Mn: 21.72 Fe: 51.42 Cu: 1.93 Zn: 45.7 ppm. In comparison, AR-120 showed similar results, with concentrations of Mg: 7,642.04; P: 5,372.42; S: 8,832.69; B: 3.55; Mn: 26.89; Fe: 55.46; Cu: 2.41 and Zn: 57.98 ppm (Figure 3A and 3B).

Mustard exhibited an increasing behavior for the macro mineral K, where MU-0 showed (K: 55,567.91 ppm), and MU-30 (K: 69,606.72 ppm), with a decrease amount in group (K: 57,240.43 ppm). The other minerals analyzed showed similar behavior, with no significant changes as the supply of Li increased, where MU-0 had Ca: 16,103.04 Mg: 6,068.28 P: 5,390.13 S: 5,029.76 B: 0.22 Mn: 24.9 Fe: 54.03 Cu: 6.14 Zn: 61.23 ppm and MU-120 containing the number of minerals at Ca: 15,152.52 Mg: 7,359.4 P: 6,007.2 S: 6,044.09 B: 0.21 Mn: 38.42 Fe: 50.04 Cu: 3.54 Zn: 77.19 ppm (Figure 3A and 3B).

Purple cabbage behaved similarly in terms of macro minerals to arugula, but the potassium values for PC-0 remained at K: 51,440.64 ppm, while PC-120 obtained a value close to K: 50,403.76 ppm. The calcium present in species increased in PC-60, with Ca: 20,894.74 ppm, when compared to PC-0 which remained at Ca: 19,349.79 ppm, despite this fact, at the highest dosage of Li applied, the calcium values decreased with PC-120 presenting Ca: 18,495.88 ppm. Zinc and manganese increased in amounts since PC-0 had values of Zn: 74.59 Mn: 55.21 ppm, and PC-120 had Zn: 118.35 Mn: 78.46 ppm. The other minerals remained stable regardless of the lithium supply, such as in PC-0, where (Mg: 9,087.53 P: 54.76 S: 6,308.87 S: 13,004.83 B: 0.24 Fe: Cu: 3.28 ppm) of minerals was observed, while PC-120 showed (Mg: 10,236.44 P: 7,082.55 S: 15,115.41 B: 0.28 Fe: 54.83 Cu: 3.12 ppm) (Figure 3A and B).

Figure 3B illustrates that the species exhibited comparable behavior, with elevated Li assimilation as the mineral supply increased. This evidence suggested a dose-dependent relationship with biofortification. Purple cabbage had the lowest Li assimilation capacity among the biofortified groups, (70.57 ppm) in 15 group and (485.58 ppm) in 120 group. The highest values were found in arugula (112.13 ppm) in 15 group and (688.77 ppm) in 120 group and kale with (81.11 ppm) in 15 group and (722.43 ppm) in 120 group. Among the analyzed species, mustard showed the highest Li assimilation capacity, with (107.01 ppm) in group 15 and (860.55 ppm) in 120 group (Figure 3A and 3B).

Considering the agronomic data, phenolic compounds, antioxidant activity and Li accumulation, the analyses of principal components (ACP) showed that arugula was the most promising species in the study, as it had the highest phenolic content and antioxidant activity (ABTS and FRAP). It was also the second species with the best agronomic performance, after mustard (Figure 4). In addition, the concentration of 60 mg. L⁻¹ of Li in the fertilizer was chosen, since it was the concentration that showed the best agronomic results and increased the bioactive compounds in this species.

Effect on neuroprotection in Drosophila melanogaster

To understand whether arugula extract promotes any poison effect in animal models, we fed larvae of *D. melanogaster* for 5 days. During the metamorphosis stage, increasing concentrations (0.1666 - 0.333 mg/mL⁻¹) of Arugula extract showed pupal volume. Studies with plant extracts suggested that the increase in pupal volume is related to the healthy development of animals. However, lower pupal volume indicates a toxic effect. Our results demonstrated significant developmental toxicity from 0.166 mg/mL⁻¹ in a diet supplemented with (60 and 120 mg) and without Li. However, when larvae were exposed to 0.333 mg/mL⁻¹ of arugula extract, the pupae development improved (Table 3).

The locomotor ability of *D. melanogaster*-fed diets supplemented with arugula extracts was performed for 15 days. The second (0.249 mg/mL⁻¹

without Li) and fifth days (0.249 without Li and 0.333 mg/mL⁻¹ 120 mg Li). Also, on the tenth (0.249 mg/mL⁻¹ without Li, 0.333 mg/mL⁻¹ 60 mg Li, and 0.249 mg/mL⁻¹ 120 mg Li) and fifteenth days (0.333 mg/mL⁻¹ 60 mg Li) (Table 3) had a toxic effect on the thoracic musculature of adult flies.

DISCUSSION

The observed decrease in plant growth in all species at the Li 120 concentration, as evidenced by both weight and height reductions, suggests that Li concentrations in leaves of all plant species exceeding 1,000 mg. kg⁻¹ result in necrosis of the older leaves, which indicates Li toxicity. This evidence makes it unviable to cultivate all the species analyzed at this concentration (KALINOWSKA et al., 2013). One potential explanation for the observed reduction in plant size is the reduction of calcium levels in leaves as lithium concentrations increase. Previous studies have demonstrated a correlation between reduced calcium levels in brassica species and leaf reduction, burning, and necrosis (LEE et al., 2016). Conversely, there was no significant correlation between Ca content and biofortification with Li, or with the amount of Li accumulated by the species: Biofortification ($r = -0.191$, $P = 0.089$) and Li ($r = -0.108$, $P = 0.340$).

The higher results for the agronomic characteristics of the species were observed in the 60 mg. L⁻¹ group, while mustard obtained the better

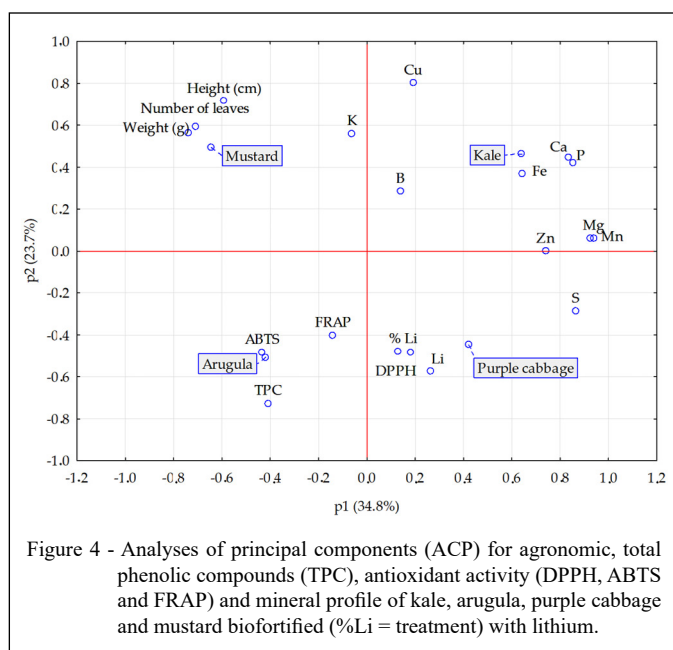


Table 3 - Axial ratio on *Drosophila melanogaster* pupae. Climbing activity of *Drosophila melanogaster* on the second, fifth, tenth and fifteenth day after eclosion. The diet standard for *D. melanogaster* was supplemented with (A) Ru0. (B) Ru60 (C) Ru120. The values represent the mean $\pm$ SEM of three experiments.

Treatment (mg.L ⁻¹ of Li ₂ CO ₃)	Axial Ratio	-----Climbing-----			
Stage	Pupal	2 nd	5 th	10 th	15 th
-----0.1665 mg/mL-----					
0	1.97 $\pm$ 0.09 ^c	0.87 $\pm$ 0.06 ^b	0.91 $\pm$ 0.02 ^a	0.91 $\pm$ 0.02 ^b	0.89 $\pm$ 0.05 ^{ab}
60	1.83 $\pm$ 0.07 ^c	0.88 $\pm$ 0.07 ^a	0.95 $\pm$ 0.03 ^a	0.96 $\pm$ 0.01 ^a	0.93 $\pm$ 0.00 ^a
120	1.92 $\pm$ 0.06 ^c	0.91 $\pm$ 0.05 ^a	0.94 $\pm$ 0.02 ^a	0.90 $\pm$ 0.03 ^b	0.90 $\pm$ 0.04 ^{ab}
-----0.24975 mg/mL-----					
0	2.39 $\pm$ 0.09 ^b	0.69 $\pm$ 0.04 ^b	0.70 $\pm$ 0.11 ^c	0.72 $\pm$ 0.06 ^c	0.77 $\pm$ 0.16 ^b
60	2.26 $\pm$ 0.04 ^b	0.94 $\pm$ 0.00 ^a	0.88 $\pm$ 0.04 ^{ab}	0.88 $\pm$ 0.03 ^b	0.94 $\pm$ 0.01 ^a
120	2.69 $\pm$ 0.05 ^a	0.92 $\pm$ 0.02 ^a	0.84 $\pm$ 0.02 ^b	0.73 $\pm$ 0.03 ^c	0.92 $\pm$ 0.02 ^{ab}
-----0.333 mg/mL-----					
0	2.88 $\pm$ 0.08 ^a	0.89 $\pm$ 0.03 ^b	0.89 $\pm$ 0.03 ^a	0.79 $\pm$ 0.13 ^b	0.90 $\pm$ 0.03 ^{ab}
60	2.76 $\pm$ 0.14 ^a	0.91 $\pm$ 0.04 ^a	0.92 $\pm$ 0.01 ^a	0.70 $\pm$ 0.09 ^c	0.88 $\pm$ 0.03 ^b
120	2.87 $\pm$ 0.03 ^a	0.95 $\pm$ 0.02 ^a	0.89 $\pm$ 0.02 ^a	0.90 $\pm$ 0.01 ^b	0.88 $\pm$ 0.04 ^{ab}

*Different letters in the column indicate that the values differ significantly according to Tukey's test ($P < 0.05$).

results for the parameters evaluated in the 30 mg.L⁻¹ group. This is due to the size of mustard, which is naturally more significant than other species. Consequently, the species contains more water than other species, which results in a dilution of minerals and compounds present in plants because the species contains more water than other species.

Upon analysis of the results obtained for the minerals, it was observed that each species exhibited varying results when observed individually. Among the analyzed species, kale exhibited the highest P content, which may contribute to its increased nutrient content, as P is considered essential for the physiological development of plants (ABDALA et al., 2015). Potassium (K) is present in large quantities in species. Potassium plays a vital role in translocating sugars and organic acids to other plant organs and regulating water loss from plants (DREYER, 2014). The highest concentrations were observed in 30 group for arugula, mustard, and purple cabbage, and in 15 group for kale, showing that Li affects the K content differently according to species.

The most significant results related to Ca were observed in kale and purple cabbage, while the lowest values were found in arugula, regardless of the biofortification level. Calcium content increased with increasing Li supply and decreased when the plants were in 120 group. This result should be considered since Ca is essential for the plant's cellular structures (WHITE, 2003). Magnesium (Mg) is present in

significant amounts in studied brassicas, with a higher content observed in 15 group compared to control. However, the relationship between Mg and Li was not dose-dependent, as there was a decrease in Mg concentration as the Li concentration was increased in vegetables for the 30 and 60 groups, followed by an increase in the highest concentration (120). The Mg content was higher in 120 group compared to the control of the respective species, which is positive since Mg deficiency can contribute to the inhibition of photosynthesis (KOBAYASHI & TANOI, 2015) and; consequently, hinder proper plant development.

Sulfur (S) content increased as the added Li content increased, in contrast to the behavior of Mg and P. Purple cabbage stood out among the analyzed species due to its high S content, which can be a positive point since S is considered the fourth essential element, after nitrogen (N), P, and K. It contributed to the synthesis of amino acids, chloroplasts, sulfides, and vitamins (MARUYAMA-NAKASHITA, 2017; NAKAI & MARUYAMA-NAKASHITA, 2020; YOSHIMOTO & SAITO, 2019). Mustard biofortified with the highest Li dose had 20% more Li in dry mass than kale and arugula in same group. Boron (B) did not show significant amounts in any species. Although, there are traces of the mineral in 0 group of kale, it does not appear in any of the other species, even though it is considered an essential mineral for vascular plants (BROADLEY et al., 2012).

Manganese (Mn) was present in small quantities in analyzed samples. The amount of Mn increased as the plant received Li. The highest levels of this mineral were observed in kale and purple cabbage. Mn is considered phytotoxic, and its primary function in plants is the activation of growth enzymes, which explains the low concentration since the plants were already in the adult phase at harvest (FERNANDO & LYNCH, 2015). Iron (Fe) is considered an immobile mineral in plant metabolism and is responsible for participating in plant photosynthesis. Low concentrations were found in all species, including the control group, in the presented mapping. An observation is that the amount of the mineral decreased from the first biofortified group and was even lower in the last group compared to the control group in case of kale, with emphasis on the 15 group where there was a significant increase compared to the other groups in all species.

The analyzed vegetables had small amounts of copper (Cu). The concentration of Cu decreased as the Li content increased in all species, resulting in a lower concentration than the control in all species when considering the 120 group. Cu aids in plant photosynthesis and disease protection at low concentrations; at high concentrations, it can be toxic (BURKHEAD et al., 2009). Zinc (Zn) participates in processes such as carbohydrate, protein, and hormone synthesis in plants (STANTON et al., 2022), and it showed increasing numbers as the Li supply increased in all species.

Regarding phenolic compounds, HEIMLER et al. (2006) conducted a study on various Brassicaceae species, where kale exhibited the highest content of these compounds, with 20 different phenolics. This conclusion is supported by the data in figure 1A, where kale in 120 group showed significantly higher total phenolic content than the control group of the same treatment. We can observe that the species' total phenolic concentrations and antioxidant activity were more evident at the higher Li concentration, which contrasts with the optimal dose for plant growth. It is noteworthy that the growth of Brassicaceae plants is influenced by the number of phenolic compounds (plant secondary metabolites), as an excessive increase indicates plant stress levels and may result in a decrease in plant size (MAGENEY et al., 2017). The dosage that resulted in the greatest increase in plant height did not demonstrate the most pronounced antioxidant effect. Conversely, the dosages with the highest antioxidant effects exhibited the lowest impact on fresh mass development. This discourages

cultivation at the highest concentration studied and highlights the benefits of intermediate Li dosages.

A study by BACHIEGA et al. (2016) examined the antioxidant activity in brassicas biofortified with selenium and observed satisfactory antioxidant capacity levels, including in the control group. This result is attributed to the fact that total phenolic compounds are the main antioxidant agents in brassicas. Therefore, higher levels of total phenolic compounds indicate a satisfactory antioxidant action. Another finding was the increase in antioxidant activity with higher levels of biofortification, which aligns with the results obtained in current research.

The pupal volume represents an essential stage in the development of most insects, including *Drosophila melanogaster*. During this stage, metamorphosis occurs, forming specialized tissues, such as flight muscles, digestive tract, and nervous tissue (TAKAOKA, 1957). Herein, larvae fed a diet supplemented with Arugula extract during metamorphosis (pupal stage) showed a reduced axial ratio ($0.1666 \text{ mg/mL}^{-1}$) but improved with 0.333 mg/mL^{-1} , suggesting a dose effect. The activity of the thoracic musculature is inherent in all animals but with specialized functions such as territorial displacement, hunting, and flight. The thoracic musculature in *D. melanogaster* specializes in terrestrial and aerial displacement. Studies have shown that the ability to fly is compromised by intoxication (IWAMOTO, 2011). Therefore, climbing ability is an important parameter to measure the effect of a diet supplemented with plant extracts (DE ALENCAR et al., 2023; SILVA et al., 2022) and molecules such as omega-3 (DE OLIVEIRA SOUZA et al., 2017). Our results demonstrated that flies fed with Arugula extracts (0.166 mg/mL^{-1} with and without Li) showed an effect against aging until 15 days. Similarly, Amazon plant extracts, *Piranhea trifoliata* (0.1 mg/mL^{-1} for ten days) (DE ALENCAR et al., 2023) and *Himatanthus succuba* (0.1 mg/mL^{-1} for 15 days) (SILVA et al., 2022) demonstrated maintenance of muscle metabolism even during the aging of animals, suggesting that ingestion of Arugula extracts as anti-aging in thoracic musculature of *D. melanogaster*.

Overall, our results suggested that the tested Brassica species can effectively be biofortified with lithium (Li), as evidenced by a progressive increase in the concentration of Li in the nutrient solution, which correlates with higher levels of the element in the edible portions of the vegetables. These findings underscore the potential of vegetable biofortification as a valuable approach for creating personalized nutrition tailored to specific populations. Lithium

biofortification could serve as a significant strategy for individuals facing mental health challenges. Additionally, research indicates that microdoses of lithium (1.5 mg of lithium carbonate per day or 0.006 mEq of lithium/kg), achievable through the biofortification methods evaluated in this study, have been shown to modify the pathological characteristics of mental disorders, with positive effects observed in both experimental and clinical settings (PEREIRA et al., 2024). This presents a promising treatment avenue for these conditions, particularly when combined with lifestyle modifications such as regular physical activity and environmental enrichment strategies that may help safeguard the brain against the onset of Alzheimer's disease and other neurodegenerative disorders.

CONCLUSION

This study demonstrated, for the first time, an increase in the antioxidant capacity of the species at higher levels of biofortification, along with a decrease in plant yield. This raises the question of whether the benefits gained outweigh the yield losses. It also highlighted the improvement in plant yield in certain species with a lower dose of Li, showing better levels of phenols and antioxidant capacity compared to the control, which suggests an enhancement of the food's properties compared to the same. Thus, to reduce possible losses in agronomic production and increase bioactive compounds and Li accumulation in the species studied, the best dosages for Li biofortification found were: 15 mg.L⁻¹ for purple cabbage, 30 mg.L⁻¹ for mustard, and 60 mg.L⁻¹ for arugula and mustard. The various tested concentrations of biofortified extracts of arugula were effective in maintaining the health of *Drosophila melanogaster* in terms of their anti-aging and neuromuscular effects in diverse assays and life stages of the animal. These findings underscore the need for further studies that elucidate the toxicity of consuming plant extracts at appropriate concentrations to understand the bioaccessibility levels of Li compared to the available Li in plants. This will expand knowledge and improve understanding in this field. Furthermore, studies on the biochemical parameters in *D. melanogaster* are necessary to provide further insights.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

DATA AVAILABILITY STATEMENT

Directly with the author.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence tools were not used to write the manuscript or to replace the authors' activities and skills, such as preparing the abstract, keywords, hypotheses and conclusion of the study.

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