Volumen 40, Nº 4. Páginas 81-90
IDESIA (Chile) Diciembre, 2022

The alkaloids of *Lupinus mutabilis* Sweet. and its use in the weed control

Los alcaloides de Lupinus mutabilis *Sweet. y su uso en el control de malezas*

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ABSTRACT

Allelopathy is described as the positive or negative effect that one plant causes on the growing of another, which occurs through the release of chemical components to the environment. The 'tarwi', *Lupinus mutabilis* Sweet., is a native legume from the Andean region whose seeds have a high protein content, however, these have a bitter taste due to the presence of alkaloids, metabolites that have been reported as allelochemical components, whose uses in agriculture are being investigated. Laboratory experiments were conducted to determine the effect of 5 concentrations (0, 1.55, 3.10, 4.65 and 6.20 mg mL^{-1}) of aqueous extract of tarwi alkaloids on the germination and growth of in vitro seedlings of three species of weeds; Amaranthus dubius, Bidens pilosa and *Medicago polymorpha*, in which the germination percentage, radicle and hypocotyl lengths of the seedlings were evaluated. An inversely proportional relationship between the concentrations and lengths evaluated for all species was considered, having *A. dubius* germination and *M. polymorpha* seedling growth as the most affected. The most significant reduction in germination and plant growth occurred at concentrations above 4.65 mg mL^{-1}.

Keywords: Allelopathy, *Lupinus* mutabilis Sweet., aqueous extract, germination, seedling growth.

RESUMEN

La alelopatía es descrita como el efecto positivo o negativo que puede causar una planta en el crecimiento y desarrollo de otra, a través de la liberación de componentes químicos al ambiente. El tarwi, Lupinus mutabilis *Sweet., es una leguminosa originaria de la zona andina cuyos granos contienen un alto porcentaje de proteínas. Sin embargo, tiene un sabor amargo debido a la presencia de alcaloides. Estos son metabolitos que han sido reportados como potenciales componentes aleloquímicos, cuyos usos en agricultura se están investigando. Se realizaron experimentos en laboratorio para determinar el efecto de 5 concentraciones (0; 1,55; 3,10; 4,65 y 6,20 mg mL^{-1}) de extracto acuoso de alcaloides del tarwi sobre la germinación y crecimiento de las plántulas in vitro de tres especies de malezas:* Amaranthus dubius, Bidens pilosa *y* Medicago polymorpha*. Se evaluó el porcentaje de germinación y las longitudes de radícula e hipocótilo de las plántulas. Se observó una relación inversamente proporcional entre las dosis y ambas longitudes evaluadas para todas las especies, siendo* A. dubius *la más afectada en germinación y* M. polymorpha *en crecimiento de plántula. La mayor reducción en germinación y crecimiento de plántulas se obtuvo en concentraciones mayores de 4,65 mg mL^{-1} de extracto.*

Palabras clave: *Alelopatía,* Lupinus mutabilis *Sweet., extracto acuoso, germinación, crecimiento de plántula.*

Introduction

The presence of weeds or unwanted plants represent a constant threat to agriculture, mainly during the establishment of the field crop because of the competition for water, light and nutrients from the soil. Furthermore, weeds have more competitive advantages over most crops in terms of biotic and abiotic stress and regarding climate change effects in agriculture. The interference of these plants can cause the reduction of yield by up to 32%, almost equal to the losses caused by pests and diseases (40%). In addition, it generates large economic losses; to avoid them, it is mandatory

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Fecha de recepción: 21 de marzo, 2022.
Fecha de aceptación: 4 de junio, 2022.

to apply an integrated weed management plan, of which chemical, cultural and biological control stands out (FAO, 2018).

Over the past 40 years, the use of chemicals in agriculture has far surpassed other methods within integrated management, becoming the main tool for weed control in most areas. However, as a consequence of the indiscriminate use of these substances, herbicide-resistant weeds germplasms are constantly increasing, leaving some farmers not only with crop losses, but with few options to fight weeds (Khan *et al*., 2019). Based on a better understanding of weed biology and ecology, the development of new weed management products and technology can lead to more sustainable weed control strategies (Westwood *et al*. 2018).

According to Olson (2015), there is a trend to increase the use of bioherbicides, expecting to overtake synthetic herbicides by 2050. Bioherbicides are closely related to the development of isolation and compound identification techniques based on plant material, having the potential to provide a sequence of benefits such as increased target specificity and immediate degradation. Nowadays there is a public concern about the harmful effects of pesticides on human health and the environment due the toxicity of its components, increasing the interest on the use of non-chemical weed management methods (Cordeau *et al.,* 2016).

Allelopathy is a biological phenomenon in which an organism's growth and development are affected by chemical substances released by another nearby organism. These substances have various bioactive metabolites called allelochemicals, that could represent an appropriate substitute for synthetic herbicides due to their low residual effect and representing an environmentally friendly option for a more sustainable agriculture. Allelochemicals can be extracted and isolated from different plant species, including lupines *(*Latif *et al*., 2017; Bhadoria, 2018).

Tarwi (*Lupinus mutabilis* Sweet.) is the only edible grain legume whose origin is focused on the Andes, mainly in Peru, under different production systems. According to the news portal Agraria.pe (2020), in 2019 tarwi national production reached 17 thousand tons, highlighting that in the last 7 years the production of tarwi has remained constant.

L. mutabilis seeds have very high protein values; however, they contain anti-nutritional substances, mostly alkaloids, which give them a bitter taste and make it difficult to consume directly, besides being toxic for human consumption. Quinolizidine alkaloids are heterocyclic secondary metabolites of an alkaline nature that are present in tarwi seeds. Several researches indicate that this metabolite has a direct effect on the germination and growth of some plants, representing an opportunity for its use as a bioherbicide (Latif *et al*., 2017; Wu *et al*., 2017).

The objective of this research is to determine the phytotoxic potential of the aqueous extract of alkaloids, obtained from tarwi seeds (*Lupinus mutabilis* Sweet) var. 'Andenes', on three important weeds on the Peruvian coast: spleen amaranth (*Amaranthus dubius*), blackjack (*Bidens pilosa*) and bur clover (*Medicago polymorpha*).

Materials and methods

The study was carried out at Cereals and Native Grains Program of La Molina National Agrarian University, Lima - Peru located at 12º04'40.1" S, 76º56'37.6" W and an altitude of 287 m a.s.l.

Plant material

Tarwi seeds (*Lupinus mutabilis* Sweet.) var. 'Andenes' were provided by the Andes Regional Development Institute (11º51'37.967" S, 75º23'48.754" W) at an altitude of 3304 m a.s.l.), obtained from the 2018 harvest. Four kilograms of tarwi seeds were ground and the flour was defatted with hexane in Soxhlet equipment.

Extraction and isolation of crude alkaloids

The alkaloid extraction process was performed following the modified protocol of Zamora-Natera *et al*. (2008). Fifty grams of defatted flour was placed in a 250 mL flask and homogenized with 200 mL of 5% trichloroacetic acid (TCA) under constant agitation for 12 hours on Brunswick Scientific G10 rotary shaker. The mixture was centrifuged in Falcon tubes for 20 minutes at 2650 x g–force in the Multifuge 3 L–R centrifuge. The supernatant was alkalinized with 8 mL of 10 M sodium hydroxide in a separatory funnel. The alkaloids were extracted with dichloromethane (3x50 mL), the organic phase was recovered and dried at 40 ºC with the Buchi R II rotary evaporator, thus separating the solvent from the crude alkaloid

extract. The extract was resuspended in methanol and transferred to amber vials, after 24 hours the solvent was evaporated and stored at 4 ºC until use in the assays.

Thin layer chromatography

Thin layer chromatography test was performed in order to verify the presence of alkaloids in the extract obtained. Aliquots of 20 µL of the extract were added on Silicagel 60 F254 plates (20 x 20 cm, 250 µm). The solvents chloroform, cyclohexane and diethylamine (6:4:1) were mixed to form the mobile phase. Dragendorff's reagent (DR) was used to reveal the stain profile, which was applied uniformly to the plate and left to dry for 3 hours in the fume hood. Alkaloids, if present, will react with DR and produce an orange stain. Once the stain profile is revealed it was compared with the one established for *Lupinus mutabilis* by Muzquiz *et al.* (1993). As a result, orange stains were spotted and the comparison of both of the stain profiles allowed the confirmation of the presence of alkaloids and the identification of two main quinolizidine alkaloids: spartein and lupanine.

Weed seed collection

Amaranthus dubius and *Bidens pilosa* seeds were collected at La Molina National Agrarian University campus in October 2018. *Medicago polymorpha* seeds were provided by the Andes Regional Development Institute, located in the Mantaro Valley of Junín region, collected on November 2018. After collection, seeds were cleaned, kept in paper bags and dry stored at room temperature (25 ºC ± 2 ºC) until germination tests were performed.

***In vitro* weed germination and seedling growth bioassays**

The crude extract of alkaloids was diluted in distilled water, concentrated in a stock solution of 200X from which the concentrations 0, 1.55, 3.1, 4.65 and 6.2 mg mL^{-1}, that were obtained in order to evaluate the effect of alkaloids on the germination and growth of weed seeds. Twenty seeds of *B. pilosa* and *M. polymorpha*, and twenty-five seeds of *A. dubius*, were placed in 9 cm diameter Petri dishes lined with Whatman Nº 1 filter paper. The Petri dishes were moistened with 4 mL of the different alkaloid extract concentration. Control Petri dishes (0 mg mL^{-1}) were also maintained in each experiment using 4 mL of distilled water. Petri dishes were incubated in darkness in the germination room, for *B. pilosa* and *A. dubius* at 26 ± 2 ºC and for *M. polymorpha* temperature was alternated, 11 ± 2 ºC for 16 hours and 24 ± 2 ºC for 8 hours. The Petri dishes were observed daily and quantitative germination data were collected until the ninth day and there was no need to water the dishes during the test. Seeds were considered germinated when the radical emerged by rupturing the seed coat. Germination percentage (daily and total), Probit analysis and germination speed index were calculated. Seedling growth, expressed in radicle and hypocotyl length, was also evaluated (Maguire, 1962; Zamora-Natera *et al.*, 2008; Algandaby and El-Darier, 2018).

Probit analysis

Probit analysis is the probability that an individual seed will germinate in a given time. Probit curve fitting is the transformation of a cumulative curve into a straight line. It was originally applied to insect survival but, under certain conditions Probit is suitable for seed analysis (Hay *et al.*, 2014).

$$Probit = logP1{-}P \quad (1)$$

Where P = percentage of germination. The probabilistic scale is graduated in units of standard deviation, which in this case correspond to cumulative percentages. The values are corrected by adding the value 5 to avoid negative numbers.

Germination speed index

Calculated by the Maguire method (Maguire, 1962; Maqueira-López *et al.*, 2021), which refers to the ratio of the number of germinated seeds to germination time. It is calculated using the formula:

$$M = nit \quad (2)$$

Where M is the germination rate, ni = number of seeds germinated on day i, t = is the germination time from sowing to germination of the last seed (total time). The higher the index, the higher the germination speed.

Statistical analysis

The experimental design was completely randomized with factorial arrangement 3A x 5B, being A the weed species factor with three species (*A. dubius, B. pilosa* and *M. polymorpha*), and B the alkaloid extract factor, with five concentrations (0, 1.55, 3.1, 4.65 and 6.2 mg mL^{-1}). Five replicates per treatment were performed. Analysis of variance was performed by test F and means were compared using Tukey's test ($p \leq 0.05$). Statistical analysis was performed using SAS® 9.3 Software.

Results and discussions

The data obtained from the analysis of variance (Table 1) at a significance level of $\alpha = 0.05$ showed that there are highly significant differences with a p-value < 0.0001 between the species, concentrations and the interaction of both.

Germination percentage

For all three species, an inversely proportional response was observed between alkaloid extract concentrations and germination percentage. However, each species reacted differently to the application of the extract (Table 2). *A. dubius* was the only species subjected to complete inhibition in germination with concentration 6.20 mg mL^{-1}. There are different levels of significance in each treatment except for concentrations 4.65 to 6.20 mg mL^{-1}, which are statistically similar.

B. pilosa showed a high germination capacity for all concentrations compared to *A. dubius* and *M. polymorpha*, whose germination percentages were notably more affected. However, with the application of the aqueous alkaloid extract there was a reduction in germination of *B. pilosa* at concentrations of 4.65 mg mL^{-1} and above, differing significantly from the control.

M. polymorpha was the species with the lowest germination percentage without applying any dose; however, germination decreased in the presence of all the concentrations applied, having a significant difference in the concentrations 4.65 and 6.20 mg mL^{-1} respect to control (Table 2).

Following the principle of allelopathy, some plant metabolites can delay or even inhibit the germination rate. Carvalho *et al.* (2019) demonstrates that ethanolic extracts of some *Amaranthus* species can led to inhibit the germination rate of *Lactuca sativa* seeds in a dose-dependent manner, the higher the concentration the lower the germination, even demonstrating a mitosis-decreasing effect with extracts of *A. spinosum* and *A. viridis*.

Germination curves by species

A. dubius showed a constant germination percentage in each of the concentrations after day two, with exception to concentration 3.10 mg mL^{-1} (Figure 1). While the germination of *B. pilosa* tends to increase in all treatments with respect to the timeline, even at the last day of evaluation, it can be observed that all concentrations exceed 70% of germinated seeds, which would indicate a good resilience capacity of this species (Figure 2). Germination in *M. polymorpha*, as with *B. pilosa*, tends to increase, but not to the same extent (Figure 3).

Table 1. ANOVA F values of germination percentage, radicle length and hypocotyl length of the three weed species against the application of the five concentrations of *L. mutabilis* alkaloid aqueous extract.

Source of Variation	D.F.	Germination percentage	Length	
			Radicle	Hypocotyl
Species	2	528.134	154.2	170.42
Concentrations	4	75.592	184.47	205.55
Species*Concentrations	8	24.717	17.56	11.03
p-Value		< 0.0001		
Coefficient of variation (%)		14.09	27.63	20.92

D.F.: degrees of freedom.

Table 2. Total germination (%) obtained per species according to the concentrations of the aqueous extract of *L. mutabilis* alkaloids at 9th-day test.

Extract concentrations	Species		
	A. dubius	B. pilosa	M. polymorpha
0.00 mg mL^{-1}	86.4 Aa	97 Aa	38 Ba
1.55 mg mL^{-1}	48.8 Bb	95 Aab	35 Cab
3.10 mg mL^{-1}	38.4 Bb	91 Aab	28 Babc
4.65 mg mL^{-1}	7.2 Cc	83 Ab	24 Bbc
6.20 mg mL^{-1}	0.0 Cc	83 Ab	18 Bc

*Means followed by the same letters, uppercase in rows and lowercase in columns, do not significantly differ by Tukey test at 5% probability.

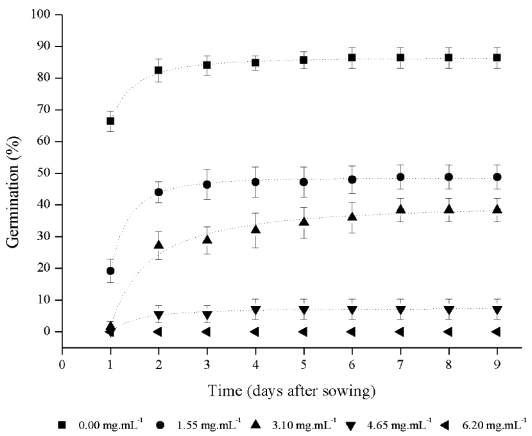

Figure 1. Germination curves of *A. dubius* according to the applied concentrations of the aqueous extract of *L. mutabilis* alkaloids.

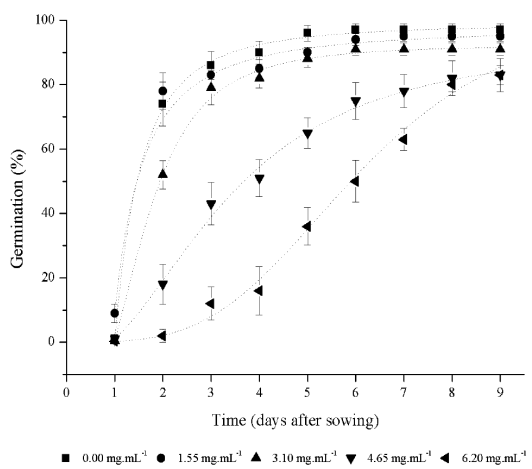

Figure 2. Germination rate curve of B. pilosa according to the applied concentrations of the aqueous extract of *L. mutabilis* alkaloids.

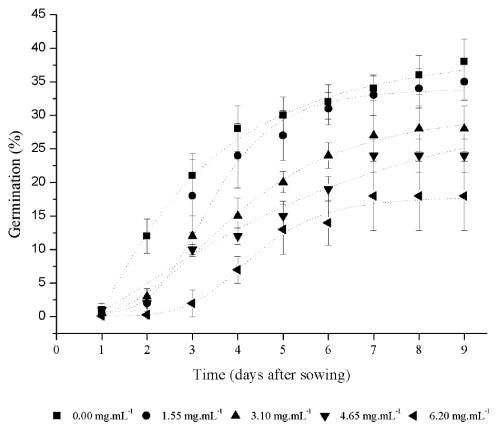

Figure 3. Germination rate curve of *M. polymorpha* according to the applied concentrations of the aqueous extract of *L. mutabilis alkaloids*.

Zamora *et al.* (2008) showed that seeds of *A. hybridus* decreased their germination due to the effect of the aqueous extract of *Lupinus mexicanus* seeds, inhibiting 80% germination at a concentration of 1 mg mL^{-1}. Thus, we can confirm the sensitivity of the genus *Amaranthus* to the application of *Lupinus* alkaloid extracts. Pannacci *et al.* (2020) also demonstrated an important decrease in the germination rate of *Amaranthus* weeds, when applying aqueous extracts of *Artemisia vulgaris.* These extracts have bioactive compounds with allelopathic activity, which allow them to have a concentration-dependent inhibitory effect on *Amaranthus.*

Corsato *et al.* (2008) observed that the extract of *Lupinus albus* affected the germination of *B. pilosa* at the lowest concentration (20%), even managing to completely inhibit the germination of the species at concentrations of 80 and 100%, which contrasts with what was obtained in this experiment, probably due to the amount of the concentrations and the species of *Lupinus* used in their research. Various studies showed that *B. pilosa* germination rate is strongly affected by other allelopathic substances as essential oils, phenolic, polyphenols and terpenoids (Dias *et al.*, 2017; Barbosa *et al.*, 2018; Ferreira *et al.*, 2020).

The effect caused by the aqueous extract of *L. mutabilis* on *M. polymorpha* can be compared to the research of Algandaby & El-Darier (2018), in which the germination of *M. polymorpha* was decreased due to the application of aqueous extracts of *Achillea santolina, Artemisia monosperma, Pituranthus tortuosus* and *Thymus capitatus*, even being totally

inhibited with *A. santolina* y *A. monosperma*, at concentrations of 20%. These species present alkaloids in their chemical composition, which confirms the sensitivity of the *M. polymorpha* to these metabolites. Ahangar *et al.* (2017) showed that germination rate of *M. polymorpha* stopped when the aqueous extract of *Artemisia sieberi* was applied at a low concentration of 25%.

Probit analysis

All treatments with different concentrations indicate a similar germination probability at day 9 (Figure 4); however, concentrations 4.65 and 6.20 mg mL^{-1} differ from 0.0, 1.55 and 3.10 mg mL^{-1} during the first days, showing a lower probability of germination until day 5, but with a tendency to increase in the following days. This reflects an achievement in the delay of germination of the species with concentrations 4.65 and 6.20 mg mL^{-1}, being more effective in the first days.

Although the lowest germination probabilities were obtained with 6.20 mg mL^{-1}, a quick recovery of germination capacity was observed with this same treatment on day 9, which means that the loss of the effect on delaying germination with 6.20 mg mL^{-1} is faster than with the other concentrations. As we can see in Figure 4, this treatment has the steepest slope compared to the other concentration lines.

Germination speed index

The germination speed index (GSI) had an inverse relationship with the concentration of *Lupinus* extract, wich decreased as the dose of extract increased (Figure 5). The data shows that *A. dubius* is the most affected species between the three weeds in its germination speed, probably due to the exposition of its peripheral embryo (Nakabayashi & Leubner-Metzger, 2021). This species had a higher delay in seed germination at the highest concentration (6.2 mg mL^{-1}) of alkaloids.

On the other hand, *M. polymorpha* has less effect on its germination speed because of its hard and thick seed testa. The reduction in germination speed indicates the effect of the allelochemical that can interfere with the establishment of the species in the environment (Oliveira *et al.*, 2004).

The germination speed index is directly affected by germination delay and, therefore, can help to significantly reduce seedling development. In this sense, as the inhibition of germination, the GSI of the treated seeds was also dose-dependently decreased (Carvalho *et al.*, 2019).

The higher concentration of the extracts delayed the germination process. The reduction in GSI suggests that the allelochemicals contained in the extracts interfere with the dynamics of the germination process. The delay in germination according to the rates ends up being an advantage

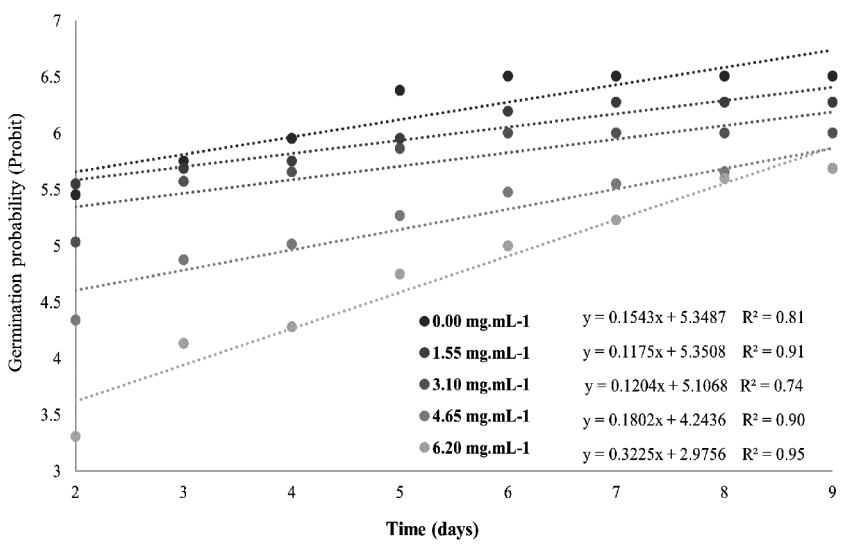

Figure 4. Probit analysis test comparing the effects on germination probability by extract concentrations of *L. mutabilis* alkaloids.

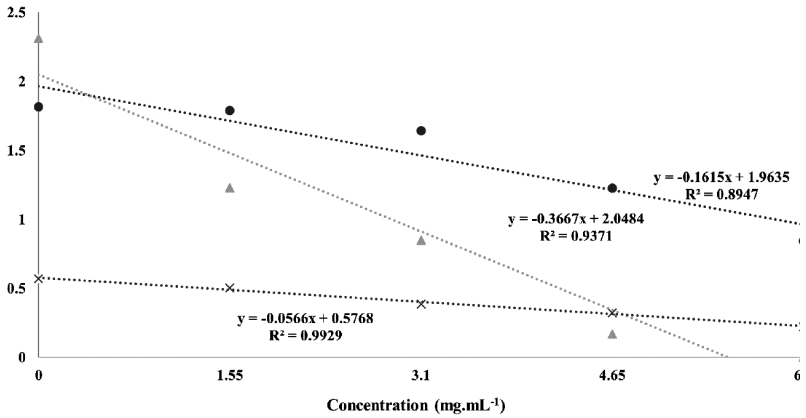

Figure 5. Gemination speed index by species (*B. pilosa*, *A. dubius*, *M. polymorpha*) for concentrations (0, 1.55, 3.1, 4.65 and 6.2 mg.mL^{-1}) of the aqueous extract of *L. mutabilis alkaloids*.

because it avoids the competition with other species (Ferreira *et al.*, 2020).

Radicle and hypocotyl length

As it is shown in Figures 6 and 7, in the three weed species, radicle and hypocotyl length were severely affected by the alkaloid aqueous extract at the concentration of 1.55 mg mL^{-1} and above.

The highest impact of *L. mutabilis* alkaloids extract on the radicle and hypocotyl length, respectively, was on *A. dubius* with a concentration of 4.65 mg mL^{-1}, this concentration can be considered as the one that produces the most significant reduction not only in germination but in plant growth. The lowest impact on the seedling emergence was on *B. pilosa* and *M. polymorpha*. Reduction of seedling growth in *M. polymorpha* under different treatments was significantly higher than *B. pilosa,* showing some tolerance of this weed against the allelochemicals contained in tarwi seeds.

Anomalies with twisted hypocotyls and root cap oxidation and discoloration were observed in extracts of 1.55 mg mL^{-1} and above concentrations.

However, it can be seen in Figures 6 and 7 that the radicle length was more sensitive than hypocotyls,

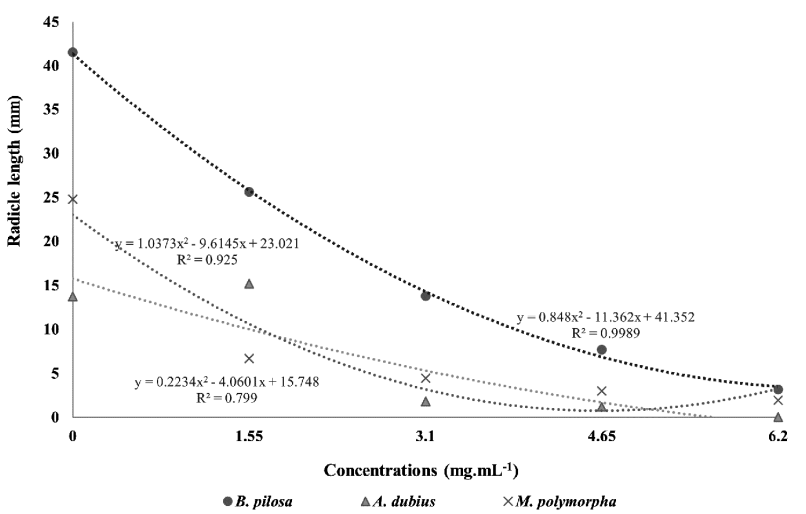

Figure 6. Radicle length by species according to the applied concentrations of the aqueous extract of *L. mutabilis alkaloids.*

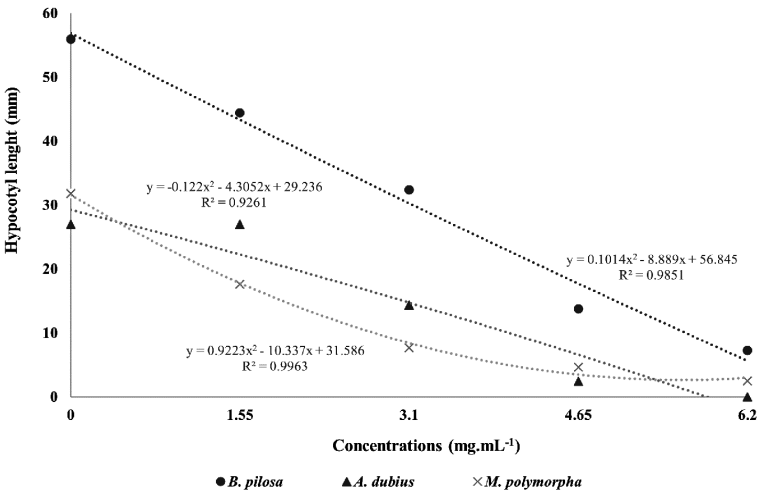

Figure 7. Hypocotyl length by species according to the applied concentrations of the aqueous extract of *L. mutabilis alkaloids.*

Table 3. Curve fitting parameters for the germination rate curve of *A. dubius*.

Equation	y = A2 + (A1−A2)/(1 + (x/x0)^p)				
Concentrations	A1	A2	x0	p	R^2
0.00 mg mL^{-1}	−4421.35	86.48	0.07	2.05	0.993
1.55 mg mL^{-1}	−3586.84	48.54	0.16	2.62	0.997
3.10 mg mL^{-1}	−2433.95	40.61	0.04	1.28	0.994
4.65 mg mL^{-1}	−37.19	7.37	0.43	1.94	0.995
6.20 mg mL^{-1}	–	–	–	–	–

Table 4. Curve fitting parameters for the germination rate curve of *B. pilosa*.

Equation	y = A2 + (A1−A2)/(1 + (x/x0)^p)				
Concentrations	A1	A2	x0	p	R^2
0.00 mg mL^{-1}	−512.26	98.88	0.45	2.08	0.9996
1.55 mg mL^{-1}	−19460.61	97.62	0.04	1.64	0.9890
3.10 mg mL^{-1}	−23.54	92.43	1.60	2.85	0.9998
4.65 mg mL^{-1}	−5.84	94.00	3.30	2.19	0.9984
6.20 mg mL^{-1}	0.13	107.75	6.15	3.53	0.9975

Table 5. Curve fitting parameters for the germination rate curve of *M. polymorpha*.

Equation	y = A2 + (A1−A2)/(1 + (x/x0)^p)				
Concentrations	A1	A2	x0	p	R^2
0.00 mg mL^{-1}	−7.54	41.87	2.52	1.70	0.999
1.55 mg mL^{-1}	0.41	34.11	3.45	4.80	0.994
3.10 mg mL^{-1}	0.00	31.28	4.04	2.96	0.996
4.65 mg mL^{-1}	−1.64	37.73	5.57	1.57	0.960
6.20 mg mL^{-1}	0.09	17.93	4.35	5.70	0.994

these results are supported by the research made by Algandaby & El-Darier (2018) in which the radicle length of *M. polymorpha* was more affected than that of the hypocotyl by the application of the aqueous extract of *Achillea santolina*, *Artemisia monosperma*, *Pituranthus tortuosus* and *Thymus capitatus*. This might be due to the fact that the radicles are the first to come into contact with the extract containing the allelochemicals.

With the results obtained, we can infer that the alkaloids present in tarwi seeds not only affect germination, inhibiting or delaying it, but also affect the physiology of the plant, mainly in the growth and development of some seedlings, with a more homogeneous effect observed in this last parameter.

The aqueous alkaloid extract of *L. mutabilis* reduced total germination and delayed the germination process. It also reduced the mean radicle and hypocotyl length by affecting the cell division and elongation processes in the different weed species, as has been reported with other allelochemicals. Radhakrishnan *et al.* (2018) state that as the weed seeds absorb the plant metabolites, this poorly initiates the cell membrane, DNA, mitosis, amylase activity and other biochemical processes and delays or inhibits seed germination. Weed growth is also slowed down due to low rates of root-cell division, nutrient absorption, photosynthetic pigment synthesis, and plant growth hormone synthesis. Carvalho *et al.* (2019) also asserted that the decrease in the mitotic index in the weed seed cells is related to the cytotoxic impact of the extract applied.

Of all the parameters measured during this study, germination can be considered the least sensitive to the alkaloid aqueous extract, whereas radicle and hypocotyl length showed more sensitivity, this is why it can be considered the most likely criteria to identify the toxicity of allelopathic compounds (Carvalho *et al.* 2019).

Conclusions

The alkaloids present in tarwi seeds affected not only the germination processes, the seedling growth and development of the weeds were affected as well. The alkaloids aqueous extract from *Lupinus mutabilis* affected the germination process decreasing the germination percentage in the three weeds species (*Amaranthus dubius* > *Medicago polymorpha* > *Bidens pilosa*). Moreover, both radicle and hypocotyl length were significantly reduced under the same treatments (*M. polymorpha* > *A. dubius* > *B. pilosa*), affecting the growth and development of the seedling. This way, alkaloids from tarwi seeds may be considered as a potential source to the production of bioherbicides.

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