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Table: 1, Figs : 2

Effects of *Tillandsia recurvata* extracts on the seed germination of *Tillandsia* spp.

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ABSTRACT

Epiphytes associate intraspecifically, but it is unclear whether the dominant species exerts a negative effect on its competitors through allelopathy. *Tillandsia recurvata* is the dominant epiphyte in the tropical dry forest of central Mexico. It has phytotoxic properties, therefore inhibits the seed germination of other epiphytic bromeliads, which may effect its associations. Nearest neighbor (NN) distance from each bromeliad was measured and the bromeliad associations were characterized as intra or interspecific. Leachates and organic extracts from *T. recurvata* were used in seed germination trials of *Tillandsia*. All species have a conspecific as their NN, but interespecific associations indicate that *T. recurvata* was less frequent NN for all *Tillandsia*. *T. recurvata* leachates and organic extracts inhibited the germination of all *Tillandsia* species. This research demonstrates the inhibitory potential of *T. recurvata* on its competitors; however its association patterns were not explained. Other ecological factors may account for its dominance and associations.

Key Words: Allelopathy, competition, dominance, interspecific associations, intraspecific associations, seed germination, tropical dry forest.

INTRODUCTION

Competition is a type of interaction that can occur by displacement or interference (15). In competition by interference, one species reduces the ability of other through, for example, the release of allelopathic compounds decrease the germination and growth of competitors (11,15). Perhaps the direct interference in the colonization or survival of competitors, occurs in communities mainly dominated by one specie. The epiphyte communities tend to associate with conspecific individuals and the interspecific associations are less frequent (3,9,23). However, at present there is no information regarding the allelopathic potential of dominant epiphytic species in influencing the associations occurring within its community.

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Tillandsia recurvata (L.) L. (Bromeliaceae) is a widely distributed vascular epiphyte (20), considered as a weed in some countries (4) and can live on inert substrates such as electricity cables (14). It can attain very high densities in relation to coexisting species in some areas (22). *T. recurvata* is a phytotoxic species (4) to the terrestrial herbs [*Medicago sativa* L. (alfalfa, Fabaceae) and *Solanum melongena* L. (eggplant, Solanaceae)] and is widely used as model species in phytotoxicity studies. One of the causes of its dominance is the reduction in germination of other sympatric species of epiphytes through allelopathic activity. If this is the case, it could be the mechanism responsible for both the high abundance of *T. recurvata* and the pattern of conspecific associations found in communities of epiphytes (3,9,23). This study aimed : (a) to describe the associations of five species of *Tillandsia* in a community of epiphytes (*T. achyrostachys, T. caput-medusae, T. schiedeana, T. hubertiana* and *T. recurvata)* and (b) to evaluate whether leachates and organic extracts of *T. recurvata* inhibit the germination of seeds of other sympatric *Tillandsia* species, and whether such inhibition can explain their associations. We predict that *Tillandsia* species must be self-associated and that if *T. recurvata* is phytotoxic to the sympatric *Tillandsia* species, then *T. recurvata* must be less frequent as the nearest neighbour of other *Tillandsia* species and the germination of this species must be inhibited by leachates and organic extracts of *T. recurvata*.

MATERIALS AND METHODS

I. Collection site and study species: The plant material was collected from San Andrés de la Cal, Tepoztlan, Morelos [(18º57'22.2'' W, 99º06'50.2'' N, 1495 m a.s.l., mean annual precipitation: 1098 mm, temperature: 12 and 18 ºC Mexico]. (16). Tropical dry forest is the dominant vegetation at the study site and is characterized by short, deciduous trees that has leaf fall from November to May. At the study site, the trees have a maximum height of 16 m, and form an open canopy. The dominant tree species include *Sapium macrocarpum* Müll. Arg. (Euphorbiaceae), *Bursera fagaroides* (Kunth) Engl., *B. glabrifolia (*Kunth) Engl. (Burseraceae) and *Conzattia multiflora* (B.L. Rob.) Standl. (Fabaceae) (22).

The community of true vascular epiphytes of this tropical dry forest is mainly composed of Bromeliaceae species [*T. achyrostachys* E. Morren ex Baker, *T. caput-medusae* E. Morren, *T. hubertiana* Matuda, *T. schiedeana* Steud., *T. ionantha* Planch., *T. makoyana* Baker, *T. recurvata* (L.) L., *Viridantha atroviridipetala* (Matuda) Espejo]. Of these species, *T. recurvata* is the most abundant with respect to the total epiphytes at the study site (72 % of individuals), followed by *T. achryrostachys* (12 %); while species like *T. hubertiana* and *T. caput-medusae* are < 4 % (22).

II. Associations among the bromeliads: In 870 m long track, at least 150 individuals of each species of *Tillandsia* were found (Table 1). From each located plant (target), its nearest neighbour (NN) was identified and the distance between them was measured. When necessary, the trees were climbed using mountain climbing equipment (10).

To determine whether intraspecific associations were more or less frequent than interspecific associations, between *Tillandsia* species, an analysis of contingency tables was conducted (8). In this analysis, the rows represented the bromeliad species for which the NN was registered and the columns represented the identity of the species of NN, while the value of the cell represented the frequency of occurrence of that association. As *Tillandsia* species differed in abundance within the study site, the expected meeting values of a NN were different from 1:1 between species and were corrected with the known abundance of species. Among the NN found, only those who were found at least 50 times were used in the analysis. The abundance ratios of the NN were 1.0:1.1:1.1:5.2:32.2 for *T. schiedeana*, *T. caput-medusae*, *T. hubertiana*, *T. achyrostachys* and *T. recurvata*, respectively. The Haberman standardized residual test was used (8) to identify which association of epiphytes was more or less frequent than expected by chance. If the Haberman residual values were more or less, than |2| the association was interpreted as significant and depending on the sign of residual, was identified as being more or less frequent than that expected by chance.

Subsequently, to know if the distance between NN determined the frequency of intra or interspecific associations, frequency histograms of each species of bromeliad were constructed with respect to the identity of its NN. In each histogram, the distances to the NN were distributed in seven categories of 10 cm in distance, and the response variable was the number of intra and interspecific associations observed, transformed as ln(frequency+0.001). With a Kendall test (18), the number of associations was correlated within each distance category to the NN. In this analysis, the distance variable denoted the class of each category.

III. Plant extracts: In November 2007, we collected 100 plants of *T. recurvata*. These plants were cleaned to remove any detritus and fauna that plants might host, chopped and oven-dried at 30^{o} C (FD 115-UL, Binder) until reaching a constant dry weight. The dry plant material was then ground to < 3 mm in an electric mill (PULVEX S.A. de C.V. model Mini-100, Mexico) until approximately 1 kg of dry material was obtained.

To determine whether *T. recurvata* has allelopathic potential on the seed germination of other bromeliad species and on its own seed germination [as done in previous studies (21)], we obtained leachates from *T. recurvata* by percolating (three times) 50 g of dried ground material with 100 ml of distilled water. Leachates were filtered through two layers of filter paper (Whatman No. 2) and used immediately.

To evaluate whether *T. recurvata* organic extracts of different chemical characteristics would have a negative effect on the seed germination of *Tillandsia* species, 800 g dried ground plant material were macerated (three periods of 72 h/solvent) at room temperature with organic solvents (hexane, dichloromethane and methanol). Following maceration, extracts were filtered and concentrated under vacuum (Rota-evaporator Buchi R-200) at 39 ºC for hexane and dichloromethane, and 46 ºC for methanol extraction. Obtained extracts were stored at -15 ºC until use.

IV. Germination trials: Seeds used in the germination experiments were obtained from capsules of 20 different individuals of each species. Capsules were collected in April and May, 2008. Seeds were disinfected with sodium hypochlorite at 0.25% and rinsed thrice with distilled water to eliminate traces of sodium hypochlorite. To determine whether *T. recurvata* would inhibit the seed germination of other bromeliad species of community (*T. hubertiana*, *T. achyrostachys, T. caput-medusae* and *T. schiedeana*) including *T. recurvata* itself, we did two germination experiments; one using leachates and the other using organic extracts.

The leachate experiment consisted of adding 3 ml of *T. recurvata* leachates to seeds of *Tillandsia* species. Twenty seeds were sown in a petri dish with filter paper (Whatman No. 2). Five petri dishes were established per species. One control treatment was implemented per species, consisting of five petri dishes with 20 seeds each, added with 3 ml of distilled water. Differences between the seed germination of each bromeliad species with *T. recurvata* leachates and distilled water were analyzed with a Student´s t test (24). To find the differences between the effects of *T. recurvata* leachates on seed germination of all the species, we employed a Kruskall-Wallis analysis of variance (18), where the principal factor was the identity of the bromeliad species and the response variable was the Inhibition (%) of seed germination. The inhibition (%) of seed germination was calculated as per the following formula (21):

$$\frac{\overline{X_0} - X_i}{n} \times 100$$

Where, $\overline{X_0}$: Mean number of germinated seeds without *T. recurvata* leachates or extracts (control), X_i : mean number of germinated seeds with leachates, i : Specific concentration of *T. recurvata* organic extracts, n : Equal to 20 seeds (sown per petri plate. A positive value indicates inhibition of seed germination, a negative value means promotion of seed germination and zero indicates no effect of *T. recurvata* leachates or organic extracts.

In experiments with organic extracts, we sowed seeds from each bromeliad species and added different concentrations (0, 0.01, 0.1, 1, 10, 100 and 1000 µg/ml) of *T. recurvata* organic extracts (hexanic, dichloromethanic and methanolic). Concentration "0" was control. Per species, each concentration within an extract had four petri dishes comprising 20 seeds each (4 replicates x 7 concentrations x 3 extracts). Filter paper was used as a substrate. Petri dishes of both experiments, leachates and organic extracts, were placed in an environmental growth chamber [(Scorpion Scientific A 50624, Mexico), photoperiod of 12 h light/12 h dark and at temperature of 30 ºC for 15 days]. The bromeliad seeds which do not produce a radicle during the germination, were considered to have germinated after the rupture of testa (7).

Statistical analysis: We did a general statistical analysis that compared the pooled effect of the three organic extracts of *T. recurvata* on the seed germination of *T. caput-medusae*, *T. hubertiana*, *T. achyrostachys*, *T. schiedeana* and *T. recurvata.* Data were analyzed with a Kruskal-Wallis analysis of variance (18), where the principal factor was the bromeliad species and the response variable was Inhibition (%) of seed germination. Multiple comparisons among medians were done with the Duncan test (18).

To test if organic extract concentration influence the inhibition of seed germination, we performed linear or quadratic regression analysis, depending on the data behavior (24). In these analysis the independent variable was the extract expressed as the ln (extract concentration + 0.001) and the response variable was the Inhibition (%) of seed germination.

When organic extracts were water insoluble, we used dimethyl sulfoxide [DMSO: $(CH_3)_2SO$, Fermont] at a maximum concentration of 1% (v/v). Previous experiments showed that seed germination with and without DMSO produced similar results in

T. recurvata (21). Compared with the control treatment (distilled water), DMSO did not have any effect on the seed germination of *T. achyrostachys* (t = 0.57, P = 0.60), *T. caput-medusae* (t = 0.92, P = 0.42), *T. hubertiana* (t = 2.61, P = 0.07) and *T. schiedeana* (t = -0.0, P = 1.00).

With a Kendall correlation test (18) we determined whether the observed percentage of intraspecific association was related to inhibition of seed germination by the *T. recurvata* leachates and the mean inhibitory effect of the organic extracts. All the analysis were carried out with the software STATISTICA 5 for Windows (Stat Soft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Bromeliads associations

Of the target species, 864 bromeliads were counted, which between them formed the principal nearest neighbors (NN). *Tillandsia makoyana* Baker and *T. circinnatoides* Matuda were also found between the NN of these species (Table 1), but these were removed from the analysis due to low frequency and the analysis was thus done with 858 bromeliads data. Associations were found between the species ($X^2_{d.\ f.\ =24}$ = 6016, P < 0.0001). With the exception of *T. recurvata*, all species were positively associated with a NN of the same species, while in all species the frequency of *T. recurvata* as NN was lower than expected by chance. Besides *T. recurvata*, no species of *Tillandsia* was consistently found to be a NN of low frequency between species.

Table 1. Identity and frequency of nearest neighbours of 5- *Tillandsia* species in the tropical dry forest of San Andres de la Cal, Tepoztlan, Morelos

Tillandsia Species	Nearest neighbour					**Total**
	T. achyrostachys (5.2)	*T. caput-medusae* (1.1)	*T. hubertiana* (1.1)	*T. recurvata* (32.2)	*T. schiedeana* (1.0)	
T. achyrostachys	**133** (25)	9 (5)	5 (6)	<u>*51*</u> (157)	<u>*0*</u> (5)	**198**
T. caput-medusae	40 (20)	**65** (4)	12 (4)	<u>*34*</u> (121)	2 (4)	**153**
T. hubertiana	15 (20)	10 (4)	**101** (4)	<u>*26*</u> (123)	3 (4)	**155**
T. recurvata	21 (26)	<u>*0*</u> (5)	2 (6)	175 (159)	2 (5)	**200**
T. schiedeana	<u>*6*</u> (19)	4 (4)	**12** (4)	<u>*37*</u> (121)	**93** (4)	**152**
Total	**215**	**88**	**132**	**323**	**100**	**858**

Parenthesis: Expected values, for abundance of each species in study area given in parentheses( ). Expected values, given below the nearest neighbour species names, are proportional values of abundance of each species in study area; this is for each individual of *T. schiedeana* there are 1.1 individuals of *T. caput-medusae* and 32.2 of *T. recurvata*, Bold: Observed values > expected, Underline: Observed values < expected, shown in italics as per standardised Haberman residuals).

For all *Tillandsia*, most intraspecific associations happened at < 20 cm and there was a negative effect of distance (τ ≥ -0.73, P < 0.05; Fig. 1). Interspecific associations had a similar pattern, which occurred most in short distances and decreased their frequency as distance increased (Fig. 1), except for *T. hubertiana*, which showed independence between distance and frequency of association (Fig. 1). Here the correlations were lower than

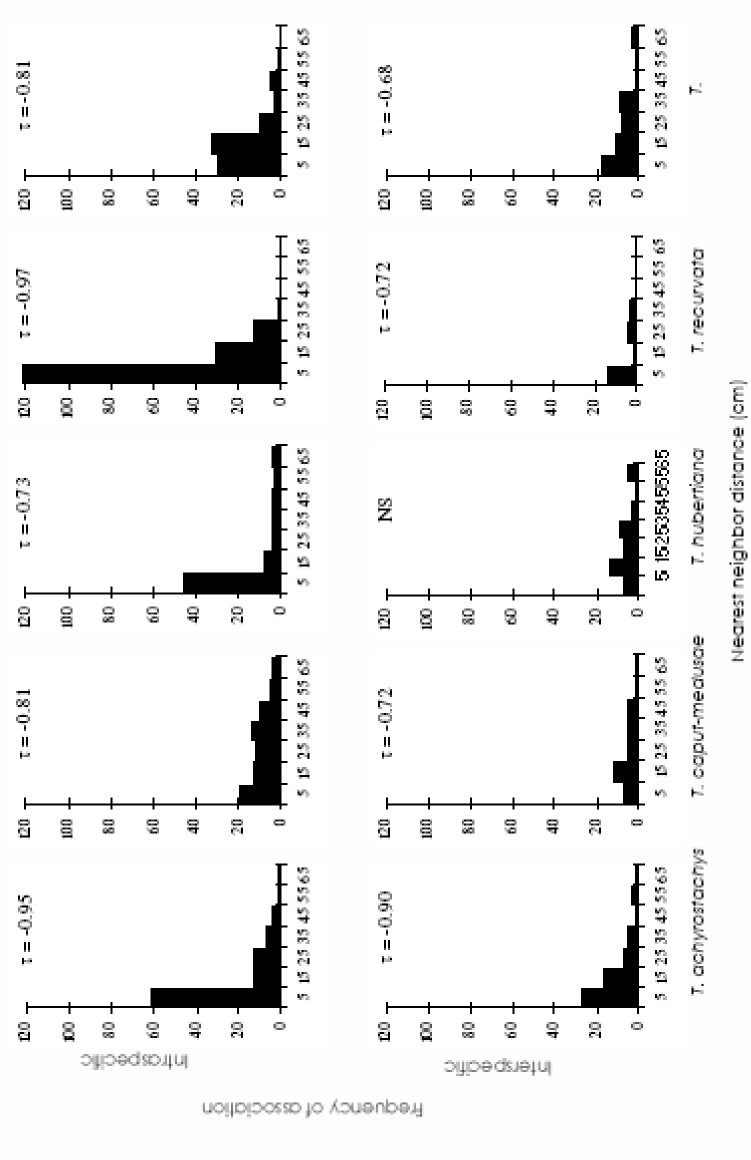

Figure 1. Relationship between the frequency of intra-specific and inter-specific associations and distance in the epiphytes *Tillandsia achyrostachys*, *T. caput-medusae*, *T. hubertiana*, *T. recurvata* and *T. schiedeana* in the tropical dry forest of San Andres de la Cal, Tepoztlan, Morelos. NS = Non significant.

intraspecific associations, *T. achyrostachys* was the species with the strongest correlation ($\tau \geq$ -0.90, P < 0.05), followed by *T. caput-medusae* and *T. recurvata* (both with $\tau \geq$ -0.72, P < 0.05; Fig. 1) and by *T. schiedeana* ($\tau \geq$ -0.68, P < 0.05; Fig. 1). At distances < 20 cm (where the majority of associations occurred), interspecific associations were less frequent than intraspecific associations (Fig. 1) (*T. achyrostachys,* $X^2_{d.\ f.\ =1}$ = 8.21, P<0.05; *T. hubertiana,* $X^2_{d.\ f.\ =1}$ = 14.52, P < 0.05; *T. recurvata,* $X^2_{d.\ f.\ =1}$ =112.02, P < 0.05; *T. schiedeana,* $X^2_{d.\ f.\ =1}$ =11.97, P < 0.05), while in *T. caput-medusae* both kind of associations were equally frequent ($X^2_{d.\ f.\ =1}$ =3.31, P > 0.05).

This supports the previous evidence, where epiphytic species associations were more intraspecific and less interspecific (9,23). This means that epiphyte distribution tends to be aggregated, as found in some terrestrial species (19). Self-association may be due to short seed dispersal distances (6,13). However, our results differ from the previous studies (23) indicated that the expected values were corrected because the abundance of species within the community was known, and they show that the most common associations were at random and that only *Tillandsia recurvata* as NN was encountered less often than expected by chance. This evidence may support the notion that allelopathic interactions could occur among *Tillandsia* species, because *T. recurvata* is the most abundant epiphyte in this forest.

Effects of Leacheates

The *T. recurvata* leachates significantly reduced (P < 0.05) the seed germination of all species of bromeliads included in this study (*T. achyrostachys* t = 5.1, *T. schiedeana* t = 3.4, *T. recurvata* t = 8.7, *T. caput-medusae* t = 3.7 and *T. hubertiana* t = 3.3) than control. Inhibition of germination by *T. recurvata* leachates did not differ between species ($H_{4,\ N = 25}$ = 8.28, P = 0.08); *T. hubertiana* was inhibited 24% ± 4.8% (hereafter mean ± 1 S.E.), *T. achyrostachys* 18% ± 1.2%, *T. schiedeana* 16% ± 3%, *T. recurvata* 15% ± 1.2% and *T. caput-medusae* 9% ± 2.4%.

It has been suggested that dominant species might influence their competitors by precipitating more efficient survival strategies (11). While it is known that the roots of the bromeliads are used for physical support only (2), and no competition exists for nutrients, these plants may still develop competitive mechanisms of interference for space. Moreover, it has been reported that *T. recurvata* is a potentially allelopathic species (4); therefore it could have a negative impact on the sympatric bromeliad species. In this study, *T. recurvata* leachates equally inhibited the germination of 5-species of bromeliads, while the extracts were more effective, but more selective, particularly affecting *T. schiedeana* and *T. achyrostachys*. In fact, more than 20% inhibition of germination only occurred in seeds of these species with the organic extracts and in *T. hubertiana* seeds with the leachates. The seed germination reduction of sympatric species could be a mechanism of competition, especially in *T. schiedeana*, which, at the study area, is the species morphologically most similar to *T. recurvata*, making it closer competitor for space. The self inhibition of seed germination could be a mechanism for controlling overload of bromeliads inside a single branch, but this needs to be investigated, because *T. recurvata* could achieve high loads on some trees (4). Although *T. recurvata* is the most abundant species in the studied forest, the frequency of self-association of *T. recurvata* was as expected by chance, supporting not self-facilitation and could be maintained by a mass effect.

Effects of organic extracts

I. ***T. achyrostachys*** : Its seed germination depended on the extract concentration of *T. recurvata*. When concentration of hexane ($F_{1, 26} = 4.2$, $P < 0.05$) and methanol ($F_{1, 26} = 5.6$, $P < 0.05$) extracts increased, germination of *T. achyrostachys* seeds diminished linearly (Fig. 2a, c). With the increase in dichloromethane extract ($F_{1, 26} = 5.5$, $P < 0.05$) concentration, germination inhibition of *T. achyrostachys* also increased but reached a maximum and there after the seed germination decreased (Fig 2b).

II. ***T. caput-medusae*** : Its inhibition of seed germination displayed a slightly positive linear dependence with the concentration of hexane extract of *T. recurvata* ($F_{1, 26} = 6.31$, $P < 0.05$) (Fig. 2d). But the germination of *T. caput-medusae* seeds was not affected by the different concentrations of dichloromethane ($F_{1, 26} = 2.1$, $P = 0.15$) and methanol ($F_{1, 26} = 0.65$, $P = 0.4$) extracts (Fig. 2e, f).

III. ***T. hubertiana*** : Its seed germination had a similar pattern to *T. caput-medusae*. Its seed germination was slightly affected by the concentration of hexane extract of *T. recurvata* ($F_{1, 26} = 4.8$, $P < 0.05$) (Fig. 2g), where inhibition of its germination increased with the increment of hexane extract concentrations. At the other hand, neither dichloromethane ($F_{1,26} = 1.8$, $P = 0.2$) nor methanol ($F_{1,26} = 2.7$, $P = 0.1$) extracts from *T. recurvata* reduced the germination of *T. hubertiana* (Fig. 2h, i).

IV. ***T. recurvata*** : Its three extracts affected its own seed germination. Concentrations of hexane ($F_{1, 26} = 5.9$, $P < 0.05$) and methanol ($F_{1, 26} = 6.9$, $P < 0.05$) extracts exerted a quadratic pattern on the inhibition of seed germination of *T. recurvata* (Fig. 2j, l). But the inhibition of seed germination of *T. recurvata* increased with the increment of concentrations of dichloromethane extract ($F_{1, 26} = 12.0$, $P < 0.05$) (Fig. 2k).

V. ***T. schiedeana*:** Its seed germination was inhibited with the increase in concentrations of hexane ($F_{1, 26} = 12.9$, $P < 0.05$) and dichloromethane ($F_{1, 26} = 11.3$, $P < 0.05$) extracts from *T. recurvata* (Fig. 2 m, n), but methanol extract did not exert any effect on the germination of *T. schiedeana* ($F_{1, 26} = 0.4$, $P = 0.7$) (Fig. 2o).

The level of inhibition of germination caused by extracts of *T. recurvata* was different between species ($H_{4, N = 360} = 199.8$, $P > 0.05$). Between species, inhibition of germination followed the gradient: *T. schiedeana* (40.3% ± 2.1%) > *T. achyrostachys* (23.3% ± 1.9%) > *T. caput-medusae* (8.2% ± 1.6%) = *T. recurvata* (6.4% ± 1.5%) > *T. hubertiana* (1.5% ± 0.6%).

The different organic extracts inhibited the seed germination of *Tillandsia* species, yet our results did not point out a particular chemical group responsible for this inhibitory activity. However, as hexane and dicholromethane extracts more frequently decreased the seed germination of different *Tillandsia* species, then chemical groups with low and medium polarity (i. e. terpenoids, fatty acids, etc.) may be affecting the seed germination of these species of bromeliads. A previous study (5) found that chloroform extracts of *T. recurvata*, which has a similar intermediate polarity as dichloromethane, contained two compounds: 1,3-di-O-cinnamoyl-glycerol and caffeic acid ethyl ester. Of

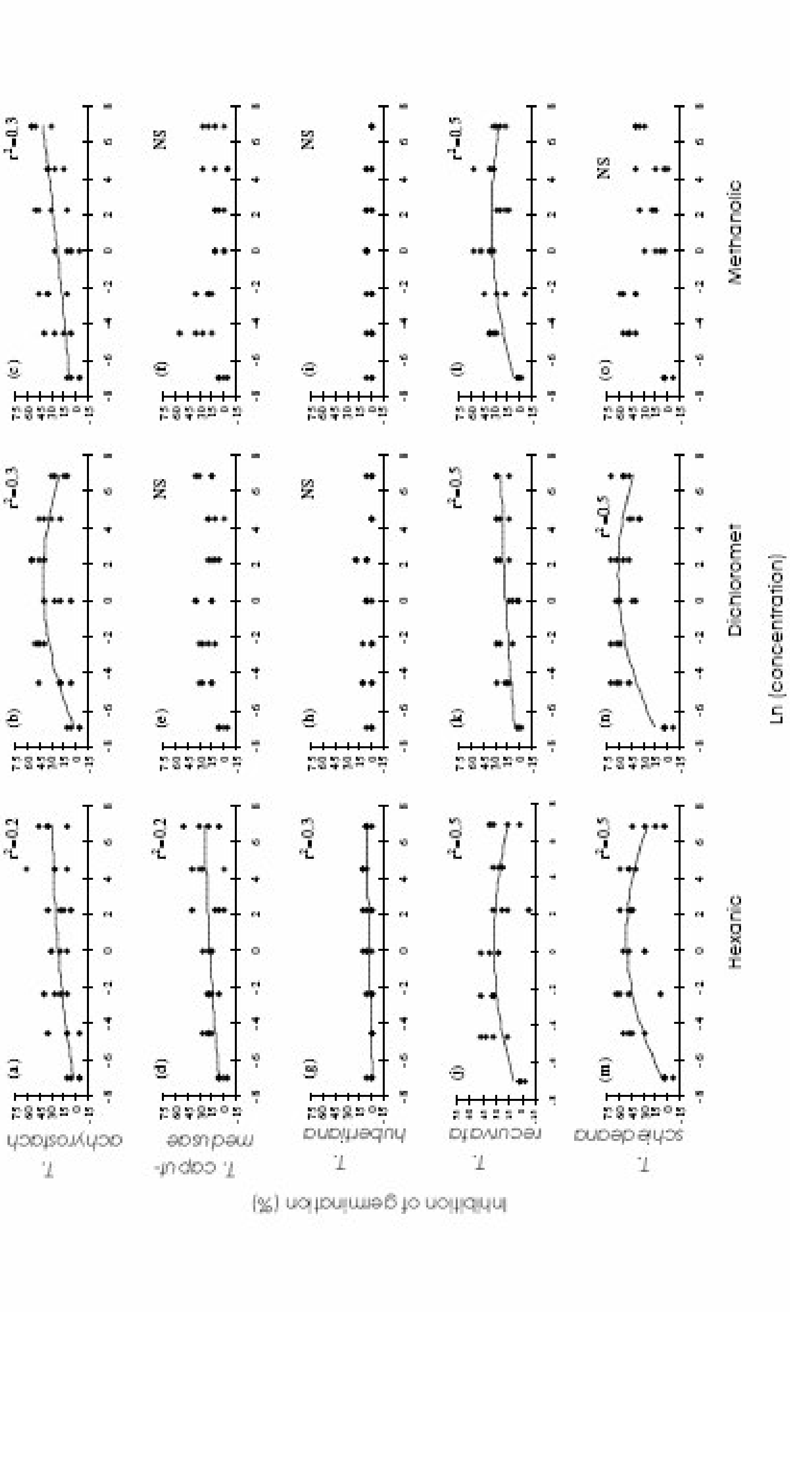

Figure 2. Inhibitory effects of organic bark extracts of *Tillandsia recurvata* on the seed germination of *T. achyrostachys*, *T. caput-medusae*, *T. hubertiana* and *T. schiedeana*. Coefficient of determination (r^2) is shown when the relationship was significant ($P < 0.05$). NS = Not significant.

these compounds, only caffeic acid has allelopathic properties (17). Due to the similarity in polarities among chloroform and dichloromethane, we did further analysis in this study, in which the dichloromethane extract was analyzed with gas chromatography-mass spectrometry. The analysis indicated that it did not contain caffeic acid but contained seven different compounds, among which only three were potentially allelopathic: phytol, palmitic acid ester and stearic acid. The diterpene phytol, in addition to forming the chlorophyll molecule, is known as a root growth inhibitor, while palmitic and stearic acids are inhibitors identified in algae and wastes in the decomposition of higher plants (1,12). However, further study would be required to show a direct relationship between the inhibition of germination of these compounds.

Relationship between inhibition of seed germination and associations with *T. recurvata*

The correlations between the probability of the five species of *Tillandsia* associating with *T. recurvata* and the inhibition of their seed germination by aqueous and organic extracts were not significant ($\tau = -0.2$ and $\tau = 0.2$, $P > 0.05$, respectively).

This is the first study to experimentally test the allelopathic potential of the dominant species in a community of bromeliads, *T. recurvata*, on the germination of seeds of five sympatric bromeliad species, including *T. recurvata* itself. However, the results of this study show independence between the inhibitory effects of *T. recurvata* extracts or leachates and the pattern of associations between the bromeliad species. It is likely that, due to the complexity of system of study, other ecological factors besides these chemical factors, determine community associations. Our measurements of species associations were done without considering plant size, as a consequence we could confound the effects of simultaneous colonization of a branch by different species, were interactions could be lowered by the small size of the seedlings. Additionally, the chemical effects of *T. recurvata* may cause interference in others life stages than germination. It is therefore important that further studies should consider these variables as influential for explaining allelopathic interactions in associations between species.

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