

**Testing the allelopathic effect of *Tithonia diversifolia* (Asteraceae) on a model species****Efecto alelopático de *Tithonia diversifolia* (Asteraceae) sobre una especie modelo**

Diana Rodríguez-Cala* and Lisbet González-Oliva

Key words: bracts, leaf aqueous leachate, leaves, lettuce germination, rhizosphere soil
Palabras clave: brácteas, germinación de la lechuga, hojas, lixiviado acuoso de hojas, suelo de rizosfera

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ABSTRACT

Tithonia diversifolia is native from Mexico and Central America. In Cuba, it is invading several regions. Particularly, it covers a large area in a protected area located at Guamuhaya cordillera: Topes de Collantes. Some authors have referred the allelopathic potential of *T. diversifolia* as the main factor that makes it so dominant in regions where it is exotic, such as Africa and Thailand. In this sense, we aim to assess the allelopathic potential of *T. diversifolia* in Cuba, through a target species with high germination and seedling emergence of *Lactuca sativa*. Three experiments were carried out covering germination and emergence of lettuce and *Tithonia*'s leaves and bracts as source of allelopathic compounds. Germination was not significantly affected by *T. diversifolia*. Radicle length was enhanced by leaf aqueous leachate, whereas hypocotyl was negatively affected. Leaves and bracts did not exerted significant effects on radicle and hypocotyl growth. Only lettuce radicle was negatively affected by *T. diversifolia*'s leaves. The rhizosphere soil and leaf aqueous leachate on soil did not exert noticeable effects on lettuce emergence. Nonetheless, hypocotyl length was slightly enhanced by rhizosphere soil. *Tithonia diversifolia* did not show strong allelopathic effects on germination and early growth of lettuce. Instead, it showed contrasting light effects depending on the source of allelochemicals and the specific target.

RESUMEN

Tithonia diversifolia es nativa de México y Centroamérica que en Cuba invade regiones como el área protegida Topes de Collantes. La alelopatía se ha considerado el principal factor que la hace tan dominante en regiones donde es exótica, como África y Tailandia. En este sentido, el objetivo de esta investigación fue evaluar el potencial alelopático de *T. diversifolia* en Cuba, a través de una especie receptora de gran capacidad germinativa como *Lactuca sativa*. Se realizaron tres experimentos que abarcaron la germinación y la emergencia de la lechuga y las hojas y brácteas de *Tithonia diversifolia* como fuentes alelopáticas. *T. diversifolia* no afectó significativamente la germinación de la lechuga. El lixiviado acuoso de hojas aumentó la longitud de la radícula, mientras que disminuyó la del hipocótilo. Las hojas y brácteas no ejercieron efectos significativos sobre el crecimiento. Solo las hojas afectaron negativamente la radícula de la lechuga. El suelo de rizosfera y el lixiviado acuoso de hojas en suelo tampoco ejercieron efectos apreciables en la emergencia de la lechuga. No obstante, la longitud del hipocótilo fue ligeramente aumentada por el suelo de rizosfera. *T. diversifolia* no mostró efectos alelopáticos notable sobre la germinación y crecimiento temprano de la lechuga. En cambio, sí mostró efectos ligeros que dependieron de la fuente de aleloquímicos y el órgano receptor específico.

* Autor para correspondencia: dianarodriguezcala@gmail.com
¹ Instituto de Ecología y Sistemática, Ministerio de Ciencia, Tecnología y Medio Ambiente, Carretera de Varona 11835

e/ Oriente y Lindero, Calabazar, Boyeros, La Habana 19, C.P. 11900. La Habana, Cuba.

INTRODUCTION

Tithonia diversifolia (Hemsl.) A. Gray is a native shrub from Mexico and Central America. It has been introduced as green manure and for preventing soil erosion in several regions of Africa, North America and Australia (Ventosa, 2011). It was introduced in Cuba for ornamental purposes (Roig, 1965), though now it is being promoted as forage (Lezcano *et al.*, 2012; Castillo-Mestre *et al.*, 2016). Due to its high propagation capacity, *T. diversifolia* has been considered invasive in many regions such as some Pacific islands, Taiwan, Thailand, South Africa, Nigeria, United States and Colombia (Global Invasive Species Database, 2011). In Cuba it is invading Sierra del Rosario (Artemisa) and Guamuhaaya cordillera (Sancti Spiritus/Cienfuegos/Villa Clara) (Ventosa, 2011). Specifically, *T. diversifolia* covers a high range in the Protected Natural Landscape Topes de Collantes, Guamuhaaya. It forms dense stands in disturbed sites where exotic species are common (Rodríguez-Cala and González-Oliva, 2015). In addition, it has been pointed out that it might displace typical tree species like *Cinnamomum montanum* (Sw.) J. Presl and *Guarea guidonia* (L.) Sleumer and herbs like *Panicum ghiesbreghtii* E. Fourn. (Rodríguez-Cala and González-Oliva, 2015).

Tongma *et al.* (2001) have referred the allelopathic potential of *T. diversifolia* as the main factor that makes this species so dominant in the mountain regions in North Thailand. In addition, Tongma *et al.* (1999) affirmed that during the dry season in North Thailand, the allelopathic substances lixiviated from *T. diversifolia*'s leaves remain at the first 5 cm in soil. Both volatile and non-volatile fractions from *T. diversifolia*'s leaves are composed by a mixture of terpenes, flavonoids and phenols (Moronkola *et al.*, 1997; Otusanya and Ilori, 2012) that have been tested as allelopathic substances (Weir *et al.*, 2004; Gniazdowska and Bogatek, 2005; Li *et al.*, 2010). In fact, several reports have been made on the allelopathical effects of *T. diversifolia* on several species (Tongma *et al.*, 1999, 2001; Taiwo and Makinde, 2005; Otusanya *et al.*, 2008; Oke *et al.*, 2011; Otusanya and Ilori, 2012; Miranda *et al.*, 2015). Taking into account this information on the allelopathical effects of *T. diversifolia* outside Cuba, this work focuses on assessing the allelopathical potential of this species in Cuba through a receptor species with high germination capacity as *Lactuca sativa* L. (Asteraceae). This receptor species might allow us to identify whether *T. diversifolia* grown in Cuba has the potential to exert allelopathical influence on native species, majority of them with lower germination capacities.

MATERIAL AND METHODS

General experimental procedures

In order to assess the allelopathic potential of *T. diversifolia*, three experiments were carried out: 1) a germination bioassay taking the leaf aqueous leachate of *T. diversifolia* as allelopathic source; 2) a germination bioassay taking fresh leaves and fresh bracts as source of volatile compounds; and 3) emergence experiment in pots with rhizosphere soil and leaf aqueous leachate of *T. diversifolia*. The fresh leaves and bracts and the rhizosphere soil were collected from *T. diversifolia*'s thickets in Topes de Collantes (21°53'N, 80°00'W, Sancti Spiritus, Cuba). The soil used as control was collected from non-invaded areas near the species' thickets. The material was transported inside wet nylon bags and stocked in cold afterwards, whereas soil was transported and stocked in nylon bags at room temperature. All the experiments were run between March and April 2015 at ambient temperature and under natural diffuse light. A completely random design was used for all the experiments.

Lactuca sativa var. BSS was used as receptor species because of its high sensibility, fast and simultaneous germination and the seed homogeneity (Fuji *et al.*, 2003; Ibrar, 2008; Morikawa *et al.*, 2012). Seeds were bought in a commercial house from 2014 harvest. We considered germination when 2 mm of radicle emerged, according to Ibrar (2008). For germination bioassays lettuce's seeds were previously sterilized with sodium hypochlorite (6%) during less than 1 minute, and then gently washed with distilled water during 1 minute.

Elaboration of leaf aqueous leachates

We sprayed distilled water on *T. diversifolia*'s fresh leaves. These leaves were put on a metallic mesh over a recipient. In order to approximate to the real rainfall in Topes de Collantes during the dry season, data were taken out from the official Management Plan of this protected area (Ruiz *et al.*, 2011). We chose the dry season for two reasons: 1) it was the period when the experiments were carried out and 2) it is the longest and more critical period in the year for plants.

We estimated the maximum threshold of distilled water volume should be sprayed so that it was equivalent to the rainfall in Topes de Collantes during the dry season. Assuming that the rainfall is the same every day and that 1 L (1000 mL) of water in 1 m² (10⁶ mm²) is equivalent to 1 mm of rainfall: $V = x \cdot y / 1000$ where V is the distilled water volume, x is the rainfall in mm and y is the area

where the rainfall is happening. In order to simulate the leaf mass that must be moistened by that rainfall, we took the maximum weight of fresh leaves produced by *T. diversifolia* per area (m²): 0.3 mg/mm² (Tongma *et al.*, 1999; 2001) and multiplied by each one of the container areas where we conducted the experiments. Each container had its own leaf aqueous leachate based on the previous calculations (Table 1). All the leachates were stocked in cold to slow down the decomposing process and applied some days afterwards.

Container	Leaf weight (mg)	Water volume (mL)
Petri dish	1908	63
Well area	1732	9
Pot area	608	20

Table 1. Leaf weight and maximum water volume resulted from the calculations for each container.

Tabla 1. Peso de la hoja y el volumen máximo de agua resultado de cálculos para cada contenedor.

Germination bioassay under leaf aqueous leachate

We put 50 lettuce's seeds per Petri dish and established two treatments: spray with distilled water (control) and spray with leaf aqueous leachate. Each treatment was replicated nine times. During the first, third and fifth days, seeds filter papers were moistened with 1 mL of the corresponding liquid (equivalent to 1 mm rainfall).

We daily counted the number of germinated seeds in order to calculate the cumulative germination percentage. Radicle and hypocotyl length were measured on the eighth day.

Germination bioassay under leaves and bracts effects

In order to evaluate the effect of *T. diversifolia*'s volatile compounds on lettuce seed germination, we performed the dish pack technique stated by Fujii *et al.* (2005) and established three treatments: i) fresh leaves ii) fresh bracts and iii) without vegetal material (control). Three dishes were used per treatment, which represented 15 replicas for treatments with leaves and with bracts, and 18 for control. Ten lettuce's seeds were put on each well. Leaves and bracts were replaced when they showed withered signals (blackish or brownish colours).

We daily counted the number of germinated seeds in order to calculate the cumulative germination percentage. Radicle and hypocotyl length were measured on the fifth day.

Pot experiment

Three treatments were established: i) rhizosphere soil of *T. diversifolia*, ii) soil of non-invaded field (control) and iii) soil of non-invaded field irrigated with leaf aqueous leachate. This last treatment (iii) was included in order to evaluate the effect of leaf aqueous leachates using non-sterilized substratum.

One lettuce's seed was sown in a pot with 20.25 cm² area and 4 cm depth. Each treatment had 60 replicas. Daily irrigation was carried out with 7 mL per replica (equivalent to 3 mm rainfall) during the first five days. Since sixth day irrigation was done twice a day (7 mL before midday and 5 mL afternoon) due to high temperatures (> 30°C).

The emergence day, the number of emerged seedlings and the number of survivable seedlings at the end of 11 days, were recorded. Radicle and hypocotyl length were measured.

Data analysis

Mean and standard error were calculated for each variable. For all the experiments, Monte Carlo tests were used to compare each variable between treatments (using PopTools 3.23 add-in of Microsoft Excel) because it does not require the assumptions of residuals normality and homocedasticity, it is based on randomization and it allows to base the analysis on size effect. This size effect was calculated as the mean value of control minus the mean value of treatment. Superior and inferior confidence limits at 95% (CLsup. and CLinf.) were used as threshold to properly declare significant differences between treatments.

RESULTS

Germination bioassay using leaf aqueous leachate

On average, lettuce's seeds germination was near 60% taking into account both treatments (Fig. 1). The germination percentage of lettuce seeds was not significantly affected by *T. diversifolia*'s leaf aqueous leachate (observed size effect = 0.44; CLsup. (95%) = 3.11). Nevertheless, seeds irrigated with leaf aqueous leachate reached the half of the total germination percentage slightly faster and had a higher number of germinated seeds on the first day (observed size effect = -4.11; CLinf. (95%) = -3.78). This light effect was decreasing with time until the eightieth day, when it was compensated.

Radicle length of lettuce seedlings ranged from 25.22 ± 0.73 mm, in the case of lettuces irrigated with

T. diversifolia's leaf aqueous leachate, to 19.51 ± 0.63 mm in control lettuces. In contrast, hypocotyl length showed an opposite behaviour: 10.88 ± 0.34 mm for lettuces irrigated with leachate and 12.22 ± 0.39 mm for control lettuces. According to Monte Carlo tests, radicle and hypocotyl lengths were affected by *T. diversifolia*'s leaf aqueous leachate. Radicles were significantly lower in control seedlings (observed size effect = -5.71; CLinf. (95%) = -2.01), whereas hypocotyls were relatively higher (observed size effect = 1.34; CLsup. (95%) = 1.02).

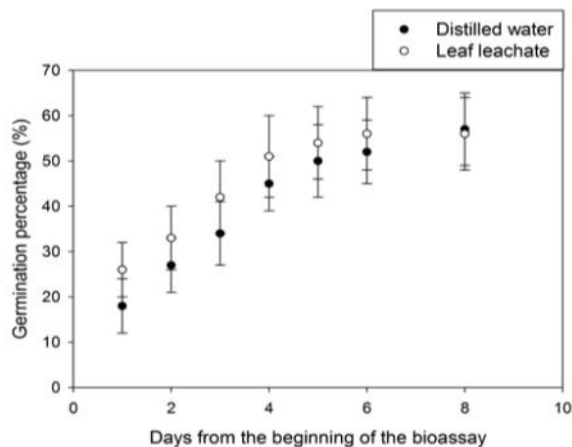


Figure 1. Germination percentage of lettuce seeds irrigated with *Tithonia diversifolia*'s leaf aqueous leachate (treatment) and distilled water (control) with error standard bars.

Figura 1. Porcentaje de germinación de semilla de lechuga regadas con extractos acuosos de hojas de *Tithonia diversifolia* (tratamiento) y agua destilada (control) con barras que representan error estándar.

Germination bioassay using leaves and bracts

In general, germination percentages were near 50% (Fig. 2). This percentage was not affected by the presence of *T. diversifolia*'s leaves and bracts as sources of volatile compounds. Nonetheless, seeds exposed to fresh leaves reached the half of total germination percentage faster and had higher values than control seeds since the third day, whereas seeds exposed to fresh bracts showed higher values than control seeds until the third day.

Radicle lengths varied from 7.85 ± 0.44 mm in seedlings exposed to fresh leaves to near 10 mm in control seedlings and the ones exposed to fresh bracts. At the same time, hypocotyl values were near 5 mm in all cases. Statistical analyses showed that radicle length was only affected by fresh leaves (observed size effect (leaves) = 2.82; CLsup. (95%) = 1.51; observed size effect (bracts) = 1.22; CLsup. (95%) = 1.62), whereas hypocotyl lengths

were not affected (observed size effect (leaves) = -0.38; CLinf. (95%) = -0.84; observed size effect (bracts) = -0.04; CLinf. (95%) = -0.90).

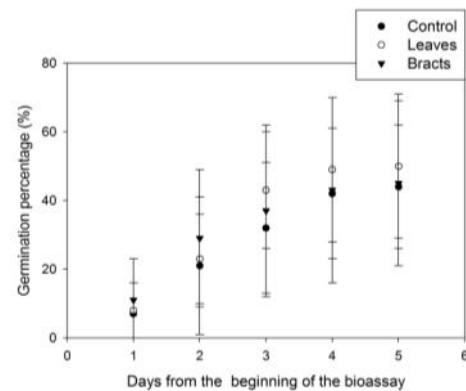


Figure 2. Germination percentage of lettuce seeds influenced by *Tithonia diversifolia*'s fresh leaves (treatment 1) and fresh bracts (treatment 2). Control has no vegetal material. Bars represent standard errors.

Figura 2. Porcentaje de germinación de semillas de lechuga bajo la influencia de hojas frescas (tratamiento 1) y brácteas frescas (tratamiento 2). El control no tiene material vegetal. Las barras representan el error estándar.

Pot experiment

Emergence percentage was 48% for both treatments and 32% for control. The lapse time for this process resulted similar for the three cases: between 10 and 11 days, with maximum values on the third day. Survival percentage was 76% for lettuce seedlings growing on non-invaded soil irrigated with leaf aqueous leachate, 89% for lettuce seedlings growing on rhizosphere soil and 84% for control seedlings. None of the treatments exerted significant effect on lettuce emergence (observed size effect (soil rhizosphere) = 0.63; CLsup. (95%) = 2.16; observed size effect (leaf leachate) = 0.15; CLsup. (95%) = 1.84).

Radical lengths were extremely low; even plants without radicle were frequently found. In addition, some lettuce seedlings developed adventitious roots. Lettuces growing on rhizosphere soil had a mean value of 1.48 ± 0.69 mm, whereas control lettuces and the ones growing on non-invaded soil irrigated with leaf aqueous leachate, had 0.94 ± 0.36 mm and 0.76 ± 0.38 mm respectively. Hypocotyl lengths varied more between treatments, though they were less than 10 mm in all cases. Seedlings growing on rhizosphere soil had a mean value of 9.67 ± 1.12 mm, whereas control lettuces and the ones growing on non-invaded soil irrigated with leaf aqueous leachate, exhibited 6.84 ± 0.89 mm and 7.85 ± 0.96 mm respectively. These variables did not substantially differ

between treatments. Nonetheless, the hypocotyl length of lettuce seedlings growing on rhizosphere soil tended to be higher (observed size effect $t = -2.84$; CLinf. (95%) = -3.52).

DISCUSSION

Effect on lettuce germination and emergence

None of the treatments: leaf aqueous leachate and vegetal material, had a significant influence on lettuce germination in general. Similar results were reported by Setia *et al.* (2007), who declared that *Bidens pilosa* germination (from Asteraceae too) was the species less affected by leaf aqueous extracts of *Eucalyptus citriodora* (Myrtaceae), which are rich in monoterpenes, allelochemicals that are also abundant in leaf and inflorescence extracts of *T. diversifolia*, (Moronkola *et al.*, 1997). However, phenols, which have been reported in *T. diversifolia*'s leachate by Otusanya and Ilori (2012), are popular inhibitors of germination (Weir *et al.*, 2004; Gniazdowska y Bogatek, 2005; Li *et al.*, 2010). In fact, Otusanya *et al.* (2008) and Otusanya and Ilori (2012) have reported the negative effect of *T. diversifolia*'s aqueous extracts on *Capsicum annum* (Solanaceae) and *Sorghum bicolor* (Poaceae) germination. In contrast, Taiwo and Makinde (2005) documented its positive effect on *Vigna unguiculata* (Fabaceae) germination. This variability in terms of effect signs (positive or negative) depending on the receptor species, suggests that the allelopathical potential of *T. diversifolia* could be species-specific (Oke *et al.*, 2011).

Nevertheless, if we analyze in particular every day during the bioassay, we can notice that the leaf aqueous leachate had a little stimulating effect on lettuce germination on the first three days and slightly increased germination speed. This pattern can be explained by two non-mutual excluding elements: 1) concentration of bioactive compounds might decrease with days due to the irrigation schedule and 2) the short mean life time of bioactive molecules might bring about a decrease in leaf leachate's biological activity during the experiment. In regard to the first statement, Tongma *et al.* (1999) reported that the allelopathic potential of *T. diversifolia* depends on the precipitations level, which is expressed as the dilution level in this case. Zhang and Fu (2010) and Kaur *et al.* (2012) said that allelochemicals can inhibit germination and growth of certain species at specific concentrations, but the same processes can be promoted as well at lower concentrations, because metabolic processes quietly depend on bioactive compounds'

concentration (Nelson and Cox, 2008). In the case of the second statement, this leaf aqueous leachate was elaborated some days before the beginning of the experiment. For a better approach, we recommend to elaborate a new leaf aqueous leachate every time seeds required irrigation, according to the schedule established by the researcher. This should be done in order to avoid the decrease of biological activity due to molecule inactivation or "aging".

At the same time, *T. diversifolia*'s leaves and bracts exerted some light stimulating effects on the last and first days of the experiment respectively. This complementary pattern could be related to the differences in withered patterns between leaves and bracts. Bracts wither faster than leaves. Another bioassay should be carried out using both leaves and bracts as source of volatile compounds, in order to assess if the effects last during the whole experiment.

The rhizosphere soil and leaf aqueous leachate of *T. diversifolia* promoted lettuce emergence, which could be due to its soil preventing capacity (Lezcana *et al.*, 2012). This capacity could be related to its allelopathical potential. However, emergence speed was not affected by *T. diversifolia* rhizosphere and leaf leachate, which can be explained because the soil microbiota uses and probably inactivates the allelochemicals exuded by roots and lixiviated by leaves (Inderjit *et al.*, 2011). This was probed in some extent by Taiwo and Makinde (2005), who reported that the compounds found in *T. diversifolia* extracts were transformed by bacterias isolated from *T. diversifolia* rhizosphere. Nevertheless, neither of those statements was actually assessed in this work.

The low germination percentages obtained in both lab bioassays could be due to aging that causes the loss of germination vigour (Bacchetta *et al.*, 2008), given to the big period that these seeds were stocked. The low emergence percentage must be related to a secondary dormancy lettuce seeds can experience when temperatures are higher than 28°C (Baskin and Baskin, 2014). Ikuma (1964) reported that lettuce (var. Grand Rapids) germination was inhibited at temperatures higher than 25°C. In addition, Thompson *et al.* (1979) documented that lettuce maximum tolerable temperature varied from 23 to 32°C, according to the lettuce variety and the sensibility to develop secondary dormancy due to high temperatures. During the emergence experiment in nursery, temperatures were higher than 30°C, and even reached more than 35°C on three consecutive days.

Effect on early growth

The inhibition of lettuce hypocotyl elongation in the treatment with leaf aqueous leachate is similar to that found in rice by Tongma *et al.* (1999). This type of inhibition has been explained as interference in mitosis and other kind of growth mechanisms by Weir *et al.* (2004) and Gniazdowska and Bogatek (2005). On the other hand, some authors such as Tongma *et al.* (1999), Otusanya *et al.* (2008), Oyerinde *et al.* (2009) and Otusanya and Ilori (2012) showed that *T. diversifolia* aqueous extracts inhibit radicle elongation in various solanaceous and gramineous species. Our results suggest stimulation of lettuce radical elongation rather than inhibition, an opposite situation with respect to lettuce hypocotyl. Opposite effects generated by *T. diversifolia* on different targets but in the same species have been already reported by Oke *et al.* (2011) and Oyerinde *et al.* (2009). However, this stimulation and inhibition of two opposite plant organs at the same time can be interpreted as an overall negative effect because normal and stable plant growth depends on a balance between underground and aboveground organs, especially during the seedling stage, the most vulnerable in plant life cycle.

The inhibition of radicle elongation exerted by *T. diversifolia* leaves is similar to the results reported by Ibrar (2008), who stated that radicle elongation of lettuce seedlings was strongly inhibited by *Tagetes minuta* leaves (from Asteraceae family too). The great amount of monoterpenes, sesquiterpenes and simple and complex phenols in *T. diversifolia* leaf ethanolic extracts (Moronkola *et al.*, 1997; Otusanya and Ilori, 2012; Miranda *et al.*, 2015) could be the answer to this inhibition. According to Gniazdowska and Bogatek (2005), the main explanation to growth reduction in plants exposed to allelochemicals is the inhibition of mitochondrial respiration (Peñuelas *et al.*, 1996; Abraham *et al.*, 2000; 2003). Nevertheless, a correlation between this molecular effect and allelochemicals photoinhibition. Perhaps, only the synergism of several compounds found in specific relative concentrations has noticeable biological activity.

The fact that *T. diversifolia* bracts did not affected lettuce early growth could be related to qualitative and quantitative differences between bract essential oil and leaf essential oil. According to Moronkola *et al.* (1997), *T. diversifolia* inflorescences have 76 volatile compounds, the double of volatile compounds found in leaves. In addition, the relative concentrations of the main compounds differ between them, which lead to substantial differences in chemical properties.

Tithonia diversifolia rhizosphere soil did not exert a significant global effect on lettuce early growth, though it slightly stimulates hypocotyl elongation. This result do not coincide with the results reported by Tongma *et al.* (2001), who conclude that soil invaded by *T. diversifolia* in North Thailand contains allelochemicals that suppress germination and early growth of other plant species. The rhizosphere soil collected in Topes de Collantes might not contain allelochemicals in sufficient amounts to exert strong effects on lettuce. Nonetheless, the secondary negative effects exerted by the high temperatures could hide the actual allelopathic effect, given to the light stimulation we observed. At the same time, the chemicals found in soil have certain life time and can be consumed by soil microbiota. In regard to this statement, Inderjit *et al.* (2011) affirmed that soil microbial activity turns biologically active chemicals into biologically non-functional molecules.

The absence of effect on lettuce seedlings when non-invaded soil was irrigated with *T. diversifolia* leaf aqueous leachates, contrasts with the stimulating effects on germination and radicle elongation and the inhibition of hypocotyl elongation that leaf aqueous leachate generated in the lab bioassay. This fact can be due to the soil microbial activity that could inactivate the chemical compounds that arrived at soil from the leaf aqueous leachates. According to Inderjit *et al.* (2011), evaluating allelopathy using a sterilized substratum is not ecologically relevant. Therefore, our greenhouse experiment complemented our previous lab bioassay and gave a more realistic approach to *T. diversifolia* allelopathy. Then, according to our results, *T. diversifolia* leaf aqueous leachate would not exert allelopathic effects on lettuce (var. BSS) early growth in field conditions. However, there are two elements that have to be taken into account: 1) as it was said before, the secondary negative effects exerted by the high temperatures could hide the actual allelopathic effect and 2) the leaf aqueous leachate was elaborated from data estimated by Tongma *et al.* (1999) in North Thailand not in Cuba.

CONCLUSIONS

In general, *T. diversifolia* did not show a strong allelopathic effect on germination and early growth of *Lactuca sativa* (var. BSS). Instead, it shows contrasting light effects depending on the source of allelochemicals and the specific target. Therefore, we cannot conclude that this species has allelopathical capacity in Cuba. We recommend testing these effects on native species coexisting with *T. diversifolia* in Cuba.

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