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IMPLICATIONS OF SEXUAL REPRODUCTION OF *TITHONIA DIVERSIFOLIA* FOR IMPLEMENTING INTENSIVE SILVOPASTORAL SYSTEMS

IMPLICACIONES DE LA REPRODUCCIÓN SEXUAL DE *TITHONIA DIVERSIFOLIA* EN LA IMPLEMENTACIÓN DE SISTEMAS SILVOPASTORILES INTENSIVOS

ROCÍO SANTOS-GALLY*

CONACYT-Instituto de Ecología, Universidad Nacional Autónoma de México, A.P. 70-275, 04510 México, CDMX.

* *Email: rsantos@iecologia.unam.mx*

Conventional cattle breeding, from pasture to free-range, is one of the primary contributors to global environmental problems, including deforestation, climate change, and biodiversity loss, thereby representing an unsustainable production form. As a response, intensive silvopastoral systems combining purposely cattle and different plant strata (pastures, grasses, shrubs, palms and/or trees) have increased in Latin America. These systems increase forage supply and soil quality, promote greater diversity in organisms, and enhance animal welfare, which involves a sustainable agricultural practice. The use of *Tithonia diversifolia* as forage shrub represents a source rich in proteins and phosphorus for cattle. Its use raises incomes, due to higher productivity. The spread of *T. diversifolia* has been conducted primarily in a vegetative form, which translates into higher implementation costs, genetic variability reduction, and plants of feebler and more superficial roots. Seed sowing could optimize its use in intensive silvopastoral systems, being crucial to identify this species reproductive condition. Likewise, getting to know fruit heteromorphism variability (two forms in the individual) at population level may provide standards for the correct planning of seed collections. This study compiles outcomes on sexual reproduction and fruit heteromorphism in *T. diversifolia,* permitting to gain knowledge on the plant and its effect on the implementation of intensive silvopastoral systems.

Key words: *Asteracea, sustainable cattle breeding, fruit heteromorphism, manual crossing, incompatibility system, pollinators*

La ganadería convencional, de pastos a cielo abierto, es una de las principales contribuyentes a los problemas ambientales globales, incluida la deforestación, el cambio climático y la pérdida de biodiversidad, por lo que representa una forma insostenible de producción. Como respuesta a esto, los sistemas silvopastoriles intensivos, que integran intencionalmente el ganado y diferentes estratos de vegetación (pastos, herbáceas, arbustos, palmeras y/o árboles), se han ido incrementando en América Latina. Estos sistemas aumentan la oferta forrajera y la calidad del suelo, fomentan mayor diversidad de organismos, y mejoran el bienestar animal, lo que conlleva a una práctica agropecuaria sostenible. El uso de *Tithonia diversifolia* como arbustiva forrajera representa una fuente rica en proteínas y fósforo para el ganado. Con su utilización se incrementan los ingresos, debido a la mayor productividad. La propagación de *T. diversifolia* se ha realizado principalmente de forma vegetativa, lo que se traduce en mayores costos de implementación, reducción de la variabilidad genética y plantas con raíces más débiles y superficiales. La siembra mediante semillas optimizaría su uso en sistemas silvopastoriles intensivos, por lo que es crucial identificar la condición reproductiva de la especie. Asimismo, conocer la variabilidad del heteromorfismo de los frutos (dos formas dentro del mismo individuo) a nivel poblacional, puede ofrecer pautas para la correcta planificación de colectas de semillas. En este trabajo se compilan resultados relacionados con la reproducción sexual y heteromorfismo del fruto en *T. diversifolia,* de manera que permitan incrementar el conocimiento sobre la planta y su repercusión en la implementación de SSPi.

Palabras clave: *Asterácea, ganadería sostenible, heteromorfismo de fruto, cruzamientos manuales, sistema de incompatibilidad, polinizadores*

Introduction

In the last decades, expansion and intensification of agriculture and cattle breeding have been recognized as main contributors to global environmental problems, including deforestation, climate change, and biodiversity loss (Gibbs *et al*. 2010). At the same time, cattle breeding is the means of subsistence for one million four hundred thousand people in the world (Herrero *et al*. 2013, 2016), and provides feed to eight hundred million people in food insecurity (Engler *et al.* 2018). Due to growing demand for animal protein (Valin *et al*. 2014), human population growth and *per capita* income

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raise (Alexandratos and Bruinsma 2012), a total of 517 million cattle and buffalo head is expected in Latin America and the Caribbean for 2050 (Alexandratos and Bruinsma 2012). Between 2010 and 2018, cattle breeding increase was related to loss of around 2,8 million ha/year in forests (FRA 2020 RSS). This pressure on ecosystems is exerted, partially, due to conventional cattle breeding practices are developed in extensive pasture areas without trees (Herrero *et al*. 2016), subject to continuous overgrazing of forages low in protein (7 %) and of low digestibility (55 %). This contributes to low cattle load (± 0.6 animal units ha^{-1}), which makes tropical cattle breeding to record poor productivity and competitiveness (González *et al*. 2015). Extensive cattle breeding promotes unproductive pastures due to soil erosion as a result of excessive grazing, low humidity retention, and high temperature due to lack of shade, so larger number of forest areas are continuously turned into new grasslands. Another factor contributing to transform land in the tropics is the flourishing commerce of feedstuffs for meat production.

Meat, as compared to other European basic food products, contributes more than 50 % to land transformation for feedstuff production (Crenna *et al*. 2019 and EC-JRC 2022). In South America, half million forest hectares are replaced for soybean production (Goldman *et al*. 2020), out of which around 77 % are devoted to forages for intensified production of animals (fattening lots) (FAO 2020). Meat supply for a growing market will promote, even more, the conversion of forests into agricultural lands for feedstuff and pasture production devoted to conventional cattle breeding.

In such context, silvopastoral systems in Latin America represent a sustainable alternative to cattle breeding, as part of the solutions based on nature to catch carbon (López-Santiago *et al*. 2019), increment profitability in cattle production (Chará *et al*. 2019), release areas non-suitable for cattle breeding to be devoted to ecological recovery (Calle *et al*. 2012), and, thus, protect part of the great biodiversity accumulated in the tropics. Silvopastoral systems consist of a well-designed combination of different plant strata such as pastures, grasses, shrubs, trees and/or palms. Particularly, intensive silvopastoral systems (SSPi) provide innovative solutions since they are based on sowing protein-rich forage plants, due to their high content of N and P, serving to be directly browsed by cattle and being a key element in the system. Moreover, electric fences are used in cattle rotation, which permits controlling soil erosion by preventing constant animal tread, whereas fresh water is provided with permanent access. Therefore, cattle are prevented to move toward natural water sources. Some plant species currently used in shrub strata in silvopastoral systems are *Leucaena leucocephala*, *Tithonia diversifolia*, *Guazuma ulmifolia*y *Sambucus peruviana* (Murgueitio *et al*. 2016). They are sown at high densities (between 5,000 and 40,000 ha^{-1}) in ranges. *L. leucocephala* promotes high nitrogen fixation and transference, whereas *T. diversifolia* favors phosphorus solubilization in acid soils. This benefits, in turn, associated pastures (Bacab 2013 and González 2013).

In Mexico, it is estimated that around ten thousand hectares of intensive silvopastoral systems of *Leucaena leucocephala* var. Cunningham have been sown in nine states of the Republic (Murgueitio *et al*. 2016 and Chará *et al*. 2019). Being native of Mexico, Central America and the Antilles (Grether *et al*. 2006), this species predominates in deciduous tropical forests with high demands of light (heliophile). It tolerates stony soils, with little resistance to flooded ones, and, above all, it grows suitably on neutral and alkaline soils (Murgueitio *et al*. 2016). That is the reason why *Tithonia diversifolia* is recommended to be sown on acid soil conditions. Also, as it is native to Mexico and Central America, this species is pioneer in different ecosystems, because it is commonly found in clearings, road verges, and anthropized places, surrounding from tropical forests up to pine-oak forests. It grows on different soils (clay, sand, lime), at 0 until 2500 m above sea level, and its use as forage species has been increased because it has a quality of up to 28.8 % protein. It is apt for different types of livestock (sheep, goat, swine, cattle) and it is widely adapted to different soils and climates (Calle and Murgueitio 2008 and Mauricio *et al*. 2017). *Tithonia diversifolia* has been used in intensive silvopastoral systems of South America and Cuba, in densities between 6 thousand and 40 thousand plants/ha. In Mexico, there are examples of its use as forage to be cut and carried in Veracruz, with promising results (Romero González 2018). Nevertheless, as part of intensive silvopastoral systems, it is still scarcely used. In the project Sustainable Cattle Breeding in the Mexican Tropics, in 2018-2020 collaborations were started with cattle farmers in the region of Los Tuxtlas, Veracruz. Currently, three ranches of dual-purpose cattle production have put in practice the vegetative reproduction of *Tithonia diversifolia* for the animals directly browse this species (figure 1). The purpose is to shift to seed sowing, so a research line has been developed to increase knowledge on sexual reproduction in this species.

The reproduction of *T. diversifola* in intensive silvopastoral systems has been developed mostly in a vegetative form (Ruiz *et al*. 2014), since seed sowing results in low germination (Zapata and Silva 2016). However, low germination has been proved in seeds sown fifteen days after collection, whereas those sown four months after being stored at room temperature (19 ºC) showed germination of 90 % (Santos-Gally *et al.* 2020) (figure 1). These outcomes corroborated latency of *T. diversifolia* in plant populations of Mexico and in plant populations introduced in Africa and Asia (Agboola *et al*. 2006, Wen 2015, Rodríguez *et al*. 2019 and Santos-Gally *et al*. 2020), which could account for the

Figure 1. Plantation of cuttings of *Tithonia diversifolia* for direct browsing in three ranches of Los Tuxtlas, Veracruz (Pictures: R. Santos-Gally).

germination percentages reported (Ruiz *et al*. 2018). Likewise, there is discrete variation in the morphological expression of the fruit (achenes) between the heads (inflorescence) of *T. diversifolia*, which is related to the fact that in some populations there are germination differences (Santos-Gally *et al*. 2020); for example, seeds derived central achenes of a population from southern Veracruz had significant differences in respect to seeds derived from peripheral achenes (87.5 % and 68.7 %, respectively) (Santos-Gally *et al*. 2020). In agricultural contexts, fruit heteromorphism can be disadvantageous because it results in germination and terraced plantation of platelets reducing yield and making harder pest management and plant growth (Mitchell *et al*. 2017). Therefore, morphos rate analysis between populations is of great relevance, particularly if wild plant material collection is planned for seed sowing.

Germination success could be also associated with sexual reproduction; that is, viable fruit and seed production that can germinate to produce the next generation. In hermaphrodite plants (presence of both sexes in the same individual), sexual reproduction is conducted by self-fertilization or cross-fertilization (Barrett 2014). Two opposing forces condition evolution in the first: advantage of transmitting 50 % of self-compatible genes and inbreeding depression. Inbreeding depression refers to reduction of viability and fertility, or both, in offspring from self-fertilization, as compared to that from crossing of genetically different individuals. Self-fertilization provides reproductive certainty, especially in ecological situations where the number of possible partners is scarce (in a colonization process or bottleneck) or where there is scarcity of pollinators (pollen grains are poorly spread) (Jarne and Charlesworth 1993). Crossing between genetically different individuals (crossing-over) promotes genetic diversity, with new alleles in a lineage that can contribute to local adaptation (Linhart and Grant 1996).

In order to ensure crossing between individuals, there are several mechanisms. The most spread in angiosperms is the system of self-incompatibility, which prevents self-fertilization (Barrett 2014). A system of self-incompatibility combines physiological, genetic (diallelic), sporophytic and biochemical mechanisms to avoid self-fertilization, which promotes exclusive fertilizations if pollen is transferred successfully between individuals (Lloyd and Webb 1992). In allogamous species (crossed pollination), viable seed production would be determined by pollen transference between genetically different partners. Determining seed production by self-pollination or crossed pollination permits establishing the presence of an incompatibility system. Due to the importance of *T. diversifolia* for implementing silvopastoral systems by seeds, this research compiles data referring to the success of seed production after various treatments which served to determine whether *T. diversifolia* has a system of self-incompatibility or not.

Development

Fruit heteromorphism

During October and November in 2019, between three to ten flowerheads were collected, out of 3-10 individuals of 32 populations of *T. diversifolia* in Veracruz, Oaxaca and Chiapas. Achenes were classified as central when they had crown with subequal and peripheral edges and crown with absence of edges (after Blake 1921). Out of a total of 28,270 achenes from 182 individuals, 12,242 central and 16,028 peripheral achenes were characterized. In total, seven populations tended to central achenes, 13 populations tended to peripheral achenes and twelve had a similar ratio of central and peripheral achenes (figure 2).

Incompatibility system

During March in 2020, manual pollinations were performed to determine if *T. diversifolia* has a system of incompatibility. Two groups of manual pollinations were set out: self-pollination and crossed pollination in 4,994 and 1,525 flowers from 29 individuals, respectively. Ten thousand and twenty-eight (10,028), and two thousand two hundred and forty-six flowers (2,246) from 29 and 9 individuals were divided into control and autonomous self-pollination groups. Flowers in the crossed pollination group were emasculated prior to the dehiscence of the anthers. The four groups were randomly assigned to four lots in different plant positions. The number of repetitions per group was balanced among the individual.

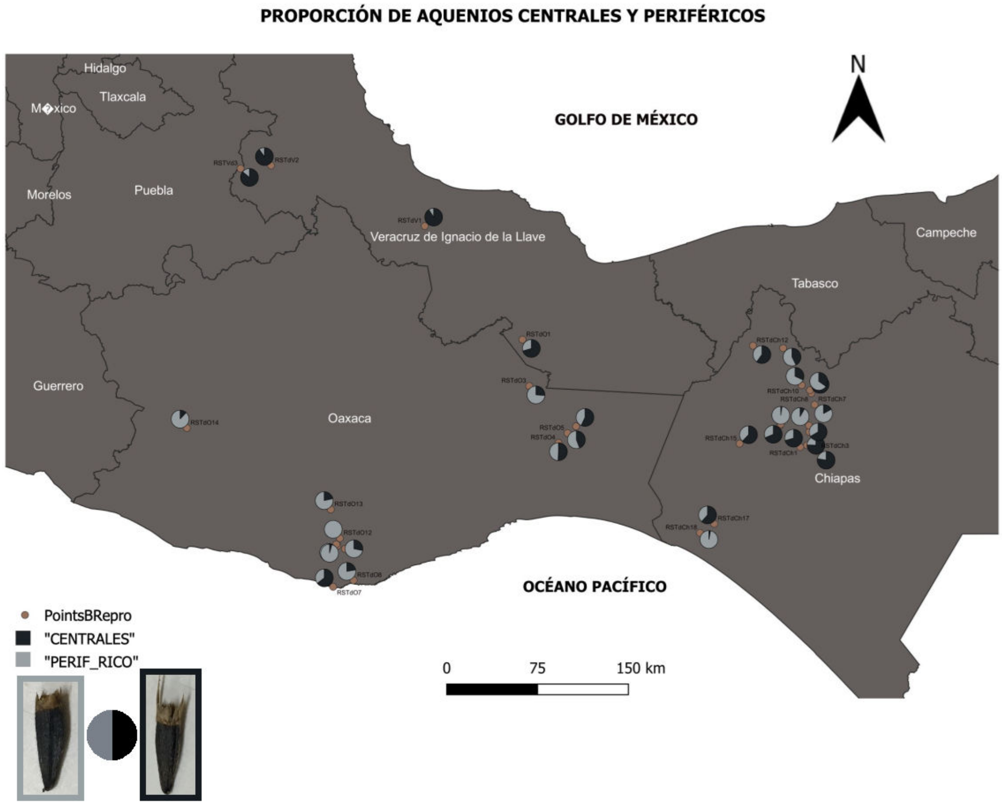

Figure 2. Ratio of central (black) and peripheral (light gray) achenes in 32 populations of *T. diversifolia* in the course of their natural distribution in Veracruz, Oaxaca and Chiapas.

Manual pollinations were performed daily for three days in lots that were marked and bagged with nets (pore size of 0.1 mm) to prevent any possible pollen contamination. Crossed pollinations involved only on donor of pollen, chosen randomly to transfer pollen to all the bloomed flowers in the lot. Self-pollination did not include emasculated flowers, which were pollinated by hand using pollen from the same flower. In both groups, flower buds surrounded flowers were removed to avoid confusion when collecting the achenes. The control for open pollination involved lots marked by buds that bloomed some days previous to or during the trial, and these buds remained available to visitors throughout the trial. With the achenes/seeds produced by these lots, it was possible to determine pollen limitation in the population or if manual pollinations were effective, which could account for the lack of significant differences between crossed pollination and the control. Finally, nine individuals from 25 lots were bagged to determine whether plants might bear fruit through non-manipulated self-pollination (spontaneous). Fruit was harvested three or four weeks after pollination, when the number of achenes with seed and aborted seed (empty achene) was counted. In order to test the effect of the different groups on fruit production (flower that turns into fruit), a beta regression model was used with beta distribution and logit binding functions. The group was included as categorical explanatory variable. For both analyses, betareg package (Simas and Rocha 2006) was used in R (Team 2018). The ratio of averages of self-pollination and crossed pollination groups was used to measure the index of self-compatibility proposed by Becerra and Lloyd (1992). Values equal or inferior to 0.75 show that species are self-compatible.

The outcomes showed that *T. diversifolia* had a system of incompatibility. The value of the self-compatibility index was lower than 0.75 (SCI = 0.55). Figure 3 shows spontaneous pollination and self-pollination had significantly lower success, as compared to crossed pollination and the control (P <0.0001). Average ratio of fruit produced per pollination was 65.31 %, whereas in spontaneous self-pollination it was 2 % (figure 3). The control did not differ significantly from the group of crossed pollination; therefore, it might be stated that there was no limitation due to pollen. The non-manipulated self-pollination group showed that *Tithonia diversifolia* demands biotic vectors to transfer male gametes and, thus, ovule fertilization.

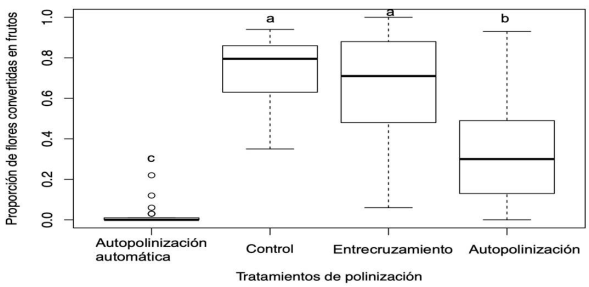

Figure 3. The box diagram shows the median of the fruit production in the four groups of pollination in *Tithonia diversifolia*. Inferior (Q1) and superior quartiles (Q3) represent measures within the range of 9-91percentile. Data of the Q1-Q3 range are represented as atypical values.

Conclusions

In different countries, *Tithonia diversifolia* is of particular interest because of its high protein content (28 %) and due to the fact that it is able of fixating phosphorus through the association with mycorrhiza, which helps soil recovery and enhances productivity (Mahecha *et al*. 2007 and Rivera *et al*. 2011). In addition to these properties, its response capacity to herbivory turns it into an excellent candidate to be used as feed in intensive silvopastoral systems (Calle and Murgueitio 2008). However, in those countries where *T. diversifolia* has been used for such purposes, plant reproduction has been limited to vegetative reproduction due to the low success in germination. The latency found in different populations of Mexico may be broken if seeds are stored during four months before the plantation (Santos-Gally *et al*. 2020), as reported in previous studies on plants introduced out of their native distribution area (Wen 2015). Peripheral achenes had lower germination success without pre-germination treatment; therefore, they are attributed higher latency (Santos-Gally *et al*. 2020). In this research, 41 % of the populations showed deviation to peripheral achenes, whereas 22 % constitute populations with deviation to central achenes. The remaining 36 % of the populations are kept in a balance between the two types of achenes. The ratio of achenes in populations should be taken into account when planning seed collection to put into practice intensive silvopastoral systems.

The presence of a self-incompatible system in *T. diversifolia* highlights the importance of having genetically different individuals to obtain the largest number of viable seeds. If choosing to set a cultivated plot of plants to obtain seeds, it is advisable that plants in this plot come from a natural population as numerous as possible, as well as seeds be chosen from individuals between 10 and 15 meters of distance with the goal of favoring genetic differences between individuals and diminishing the risk of inbreeding depression. Although in this specific research, the presence of inbreeding depression was not analyzed in self-incompatible species (*Raphanus sativus* and *Leontodon autumnalis*). There is evidence of decline in seed production in plants produced from self-pollination (Nason and Ellstrand 1995 and Picó and Koubek 2003). Plants from crossing between relatives may present negative effects due to reduction in genetic variability, which could be expressed in production, germination, or plantelet growth decline (Cheptou *et al*. 2000).

Due to the interest in *T. diversifolia* for putting into practice intensive silvopastoral systems through seeds, its obtainment should be concentrated on populations of more than 200 individuals with the aim of avoiding the harvest of empty seeds. Moreover, it should be noted that if seeds are obtained from plants that were fertilized in a vegetative form, the resulting seeds may have a loss of genetic variability that could have non-desirable effects on the growth and production of fertile seeds. In the future, it is important to research if the decrease in genetic variability could also affect forage quality or hebivory resistance, or both. This research proves seed production through crossing-over or natural pollination is 50 % higher than through self-fertilization; therefore, seeds from natural populations in the region under study may be used to plant intensive silvopastoral systems in the future. These results also highlight the importance of natural pollinators for seed production and the successful reproduction of this species.

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