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RESEARCH NOTE

Propagation of *Sophora toromiro* through interspecific grafting to support species conservation

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Abstract

J. Espejo, M. Baeza, E. Ruiz, F. Mora, M. Gomez, and G. Montenegro. 2013. Propagation of *Sophora toromiro* through interspecific grafting to support species conservation. Cien Inv. Agr. 40(1):213-221. This report provides information on applying the cleft grafting technique in the species *Sophora toromiro* (Phil.) Skottsb., focused on its propagation, and contributes to the conservation of the genetic base of this species in the medium and long term. A germplasm collection was obtained via interspecific grafting using *Sophora cassioides* (Phil.) Sparre as rootstock, employing two variations of cleft grafting sealing. The survival of 301 *S. toromiro* grafts of the Titze line at ten months was 64% or 95%, according to the type of sealing performed. A second experiment, comparing the origin of the material, indicated that the plants of Goteborg origin showed survival values close to 63%, versus 58% for those from the Jardín Botánico Nacional de Viña del Mar (Chile). This work presents a new approach for the recovery of *S. toromiro* and other woody species that are classified as endangered or highly threatened through the cleft grafting technique, in this case, onto *S. cassioides*.

Key words: Conservation, graft, *Sophora toromiro, S. cassioides*.

Introduction

Loss of species is currently occurring rapidly, due not to natural effects, but to anthropic factors (Pimm and Raven, 2000), particularly on oceanic islands, which serve as natural laboratories and are not protected from this tendency. The genesis of these islands and their uniqueness, isolation and especially their flora and fauna mean that the conservation of their genetic resources is of interest for local as well as national and international communities (Hoffmann and Marticorena, 1987).

An example of this type of situation is provided by Easter Island, or Rapa Nui, located in the Pacific Ocean (27° 09'S; 109°27'W), 3,525 km from continental Chile. The disappearance of most of

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the species on this island was associated with the anthropic impacts of the colonizing ethnic group as well as an explosive increase of the rodent (*Rattus* spp.) population (Hunt, 2007). One of the species that is now extinct on the island is the tree *Sophora toromiro* (Phil.) Skottsb. (UICN, 2009), whose botanic description comes from 1744, provided by Johann and Georg Forster. Further chronicles and expeditions from the early 19th century (Palmer, 1870; Gana, 1870; Pinnart, 1877) reported the precarious conservation of *S. toromiro* populations, located on sidehills and in higher areas of the island, which is supported by the location of the last specimen found growing naturally, inside the Rano Kao volcano on its slopes (Skottsberg, 1920; Rodríguez *et al.* 1983).

Studies conducted by Orliac (1993) based on sedimentary pollen confirmed that this species also previously extended to the lower areas of the island, specifically to its mesic environments.

Due to the ethnobotanical importance of this species, its seeds were collected and documented by Carl Skottsberg in 1917, Efraín Volosky in 1953 and Thor Heyerdahl in 1956. The last collection was performed from the last specimen known on the island (Ibáñez *et al.* 2003). These seeds were then grown in the nursery facilities of the Göteborg Botanic Garden, which has distributed them, as well as stakes, to the most important botanic gardens in Europe, such as the Royal Botanical Gardens at Kew, the University Botanic Garden at Bonn and the Jardin Botanique Exotique at Menton (Maunder *et al*. 1999).

In Chile, specimens of four lines of *S. toromiro*, which were previously defined by Maunder *et al.* (2000), can be found at present: i) Göteborg (Got), deposited in the Universidad Austral de Chile, currently under the care and management of Forestal Mininco S.A.; ii) the collection of E. Volosky, in the Jardín Botánico Nacional de Viña del Mar de Chile (JBN); iii) a specimen planted in the Faculty of Agronomy of the Universidad de Chile by Fusa Sudzuki (Sud); and iv) specimens in Jardín Las Brujas de Talagante (Chile), referred to as Titze (Tit) ex Alemparte.

The information provided by Maunder *et al.* (2000) indicates that the lines present in Chile show genetic variability. Therefore, the offspring of these grafts are expected to have the capacity to survive in their natural habitat. However, the various restoration initiatives for this species on Easter Island have been unsuccessful thus far (Christensen and Schältzer, 1993; Ibáñez *et al.* 2001).

The recovery of this species using different techniques has been proposed by Maunder *et al.* (1999, 2000). Nevertheless, new proposals for this purpose have yet to be presented.

The grafting technique has been used by different cultures for the preservation and propagation of genotypes of interest as well as to shorten reproductive cycles. In the context of the genetic improvement of plants, there are different strategies for germplasm collection, and grafting represents a proven and efficient tool in the field of asexual propagation (Zobel and Talbert, 1988).

The objective of this research was to evaluate an *ad hoc* grafting technique for *S. toromiro* for germplasm propagation through interspecific grafts with a suitable taxonomic species, in this case, *S. cassioides*.

Materials and methods

S. toromiro scions were obtained from plants of the line of the Jardín Botánico Nacional de Viña del Mar that had grown for more than two seasons in the garden. The plants of *S. cassioides* used as rootstock came from seeds collected in the foothills of the region of Bío Bío (Chile) that were grown in speedling trays and in bags in the Los Ángeles nursery of Forestal Mininco S.A., located in the city of Los Ángeles, region of Bío Bío.

Histological observations on S. toromiro and S. cassioides

An anatomical analysis of *S. toromiro* scions and *S. cassioides* stems was performed prior to the beginning of the experiments. Thus, their taxonomic affinity of *S.cassioides* to *S. toromiro* was verified to ensure the successful fusion of the tissues from the two species. The material collected from two plants consisted of branches from *S. toromiro* and stems from *S. cassioides*, which were stored in FAA (formaldehyde, 70% ethanol, acetic acid, in ratio 18:1:1. Then, the material was dehydrated in a graded series of increasing alcohol concentrations, embedded in paraffin, cut with a microtome to a thickness of 14 microns thick and stained with safranin and fast green.

Experiment 1. Grafts with material from the Titze line

Grafts

The first experiment was performed in May 2007, in which 28 mother plants of *S. toromiro* were used. These plants were obtained from autopollinated seeds cultivated in the Jardín Las Brujas de Talagante (Chile), identified as the Titze line by Maunder *et al.* (1999; 2000). The plants were two years old at the time of the experiment and were growing in polyethylene bags. These plants provided 301 scions with different diameters; at least three scion sections, defined as apical, intermediate and basal specimens, were obtained from each branch.

Two-year-old *S. cassioides* plants were used as rootstock, which were established in a tray of 104 cavities and volume of 60cc and 5-L polyethylene bags. Pine bark was used as growing medium (substrate).

The applied grafting technique was cleft grafting, which involved making a 2.0- to 2.5- cm-deep longitudinal cut in the rootstock and the subsequent insertion of a scion with two longitudinal cuts in the end, leaving a wedge shape in this section of the scion. Two alternatives were analyzed for the sealing of the scions and the rootstock. 1) Sealing with Parafilm M® and Podexal®: Parafilm M® is a plastic film used for material sealing in laboratory, and Podexal is a systemic fungicide used to prevent attacks from fungi and other organisms during the pruning of woody material. 2) Sealing using a latex band with wax: latex bands are widely used in grafts of the genus *Pinus,* their thickness and elasticity provide adequate pressure and protection of the tissues involved (scion/rootstock). In the latter technique, a block of wax is melted and applied over the entire area of the union and tissue contact with a small paintbrush.

Rootstocks with similar scion diameter dimensions were selected to provide homogenous areas of contact. Scions with a diameter greater than 1.5 mm were grafted onto plants that showed higher development and lignification in bags, while scions with smaller diameters were grafted onto plants in seedbeds.

A total of 245 scions were subjected to grafting involving sealing with Parafilm® and Podexal®, and 56 scions were subjected to sealing with a latex band using wax. Evaluation of graft survival was performed at 10 months.

Statistical analysis

Due to the low amount of material (scions) available in experiment 1 when sealing with a latex band and wax was performed, and due to the high variance found, the survival of the grafts in the experiment was analyzed using a generalized linear model (GLM). GLMs are an alternative method of statistical analysis that can be used when the assumption of normality is unsatisfied. The use of a GLM is applied in biological experiments in which the response variable follows a general distribution known as

an exponential family (Demétrio 2001, Soares *et al*. 2005), as cited by Mora *et al*. (2008). The GLM relates the random distribution of the variable measured to the systematic portion (not at random) of the experiment with a link function. Additionally, GLMs are based on the assumption that independent observations do not present a correlation with the results. The data were analyzed in the program SAS® with the procedure GENMOD.

Experiment 2. Grafts with material from Goteborg and the Jardín Botánico de Viña del Mar

The second experiment, conducted in March 2008, consisted of the same cleft grafting technique, and only Parafilm® was used to seal the scions and the rootstock. Plants from the Goteborg and Jardín Botánico de Viña del Mar lines were grafted, using plants over four years in age, which were delivered by the Universidad Austral de Chile and the Jardín Botánico Nacional to Forestal Mininco S.A. for research purposes. Due to the lack of available material, only 72 grafts were performed. The rootstocks also came from the species *S. cassioides.*

As in experiment 1, the survival of the grafts and development of the scion were evaluated 10 months after the beginning of the experiment.

Results

Histological observations

The secondary xylem tissues of *S. toromiro* and *S. cassioides* are very similar with regard to the distribution of vessels, the xylematic fiber features, the amount and distribution of the axial parenchyma and the structure of the vascular radios (Figure 1).

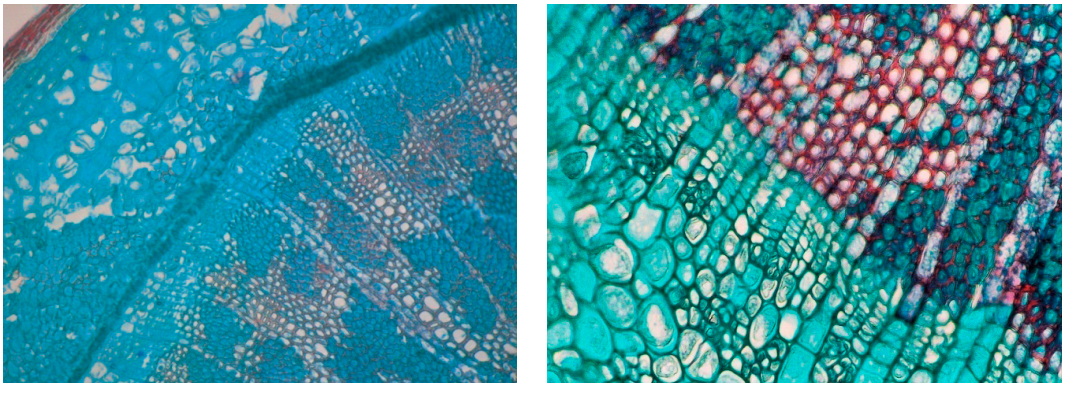

Figure 1. Cross sections of stems with secondary growth of, *Sophora cassioides* (left, 40x) and *S. toromiro* (right, 200x) showing their secondary xylem.

The analyzed samples corresponded to transverse sections of two-year-old secondary xylem from the species mentioned above, with low differentiation being observed between years. In both species, the vessels display a somewhat small diameter and slight morphological variation. They are arranged radially in irregular sets, with numerous vessels being associated with the axial parenchyma. The fibers exhibit thick walls and tangential sets. The numerous, multiserial vascular radios are strongly associated with the vessels; radios with two to three series of cells are predominant. These

histological features of the secondary xylem support the feasibility of a good seizure and union of the two types of stems in the graft region.

Experiment 1

Graft survival rates were 95% and 64% for the grafts established with Parafilm® + Podexal® and latex band + wax, respectively (Table 1). The differences in survival were found to be insignificant when the two alternative types of graft (Table 2) sealing were compared (GLM α=0.05). Although the obtained survival percentages were acceptable at the 10-month evaluation point, they should be monitored in the medium and long term.

Experiment 2

Low survival was observed in the second experiment, using only Parafilm® for graft sealing, when compared to the results from the first experiment after 10 months of grafting. A favorable response for scions from Goteborg was evident, with a mean survival of 62.5% being recorded, compared to 58.4% for the grafts from the Jardín Botánico Nacional de Viña del Mar (Table 3).

Discussion

It was found in this study that the interspecific grafting technique is feasible for *S. toromiro* using *S. cassioides* as rootstock, which will allow the exponential expansion of *S. toromiro*. A limiting factor in this study was the difficulty of obtaining a greater amount of material from *S. toromiro* lines.

Taxonomic and physiological considerations

Although taxonomic affinity was considered an important feature in this work, other factors having influence in the successful grafting techniques cannot be discarded, as supported by the histological analysis (for example, concerning the biochemical profiles of the species under

Table 1. Number of mother plants used, scions obtained, treatments applied and survival values recorded during the evaluation performed at 10 months.

Mother plants (n)	Assayed scions	Treatment	Mean (%)	Standard deviation	Minimum value	Maximum value
28	245	Parafilm® + Podexal®	95.2	9.1	60	100
	56	Latex + Wax	64.3	38.1	0	100

Table 2. Statistical results obtained with GENMOD (SAS): survival analysis

Source	2xLog(Likelihood)	DF	Chi-Square	P>Chi-Square*
Intercept	30995.1769			
Treatments	30998.8486	1	3.67	0.0553

DF: Degrees of freedom
* Treatment means were not significantly different according to a Chi-square test (analysis of deviance; α = 0.05)

Table 3. Experiments involving the provided scions.

Provenance	Assayed Scions	Minimum value (%)	Maximum value (%)	Mean (%)
Jardín Botánico de Viña del Mar	36	0	100	58
Goteborg	36	0	100	63

study). This result has been verified in studies on incompatibility in grafts *Prunus* spp. and using polyphenolic compounds Usenik et al. (2006), as cited by Gulen et al. (2002).

The high survival reported in this work may also be explained by considering that both the scion and the rootstock came from juvenile material (two years). Thus, both the degree of lignification at the cambium level and the formation of vascular tissue were still in the emerging stages, as indicated by Pina and Errea (2005).

Callus formation is variable and depends on the species. For example, in *Gardenia* spp, healing occurs between 21 and 35 days post-grafting (Mustard and Linch, 1977), while in *Prunus* spp., it takes place at between 45 and 60 days post-grafting (Oguz *et al.* 2008). The conditions of temperature and humidity affect callus formation (Oguz *et al*, 2008), which is supported by findings reported in *Abies fraseri* by Frey (2009), who examined the results of interspecific grafts. Grafting performed in winter and early spring resulted in higher survival than grafting conducted in summer.

The shading of grafts may also affects their results, as demonstrated in another experiment reported by Frey (2009), where better results were found under shading. This author recorded 52% graft survival under shade versus 38% without protection.

The response observed may be masked by the nutritional status of the mother plant, considering the origin of the material. In the present study, only a mother plant from the Goteborg line was available. It is important to note that although the obtained results are satisfactory, a long-term follow-up study is required, as incompatibility may manifest in a belated manner (Vera *et al.* 1997; Sharma and Uniyal, 2003).

Conservation of the species

Experience regarding the use of interspecific grafts in species with conservation problems is scarce. In the species *Kokia cookey*, which became extinct in 1915 on the island of Molokai (Hawaii, United States), Sugii and Lamoureaux (2004), reported a situation similar to that of *S. toromiro* on Easter Island. One plant was obtained from four seeds from the last specimen of *K. cookey*. This plant generated a great number of offspring, but they did not succeed in reintroduction. A subsequent graft of this species with *K. kauaiensis* generated twenty-three ramets (which were introduced to the island of Molokai, although flowering has not been recorded to date.

Aparicio *et al.* (2009) recently performed analyses of vegetative propagation in *Austrocedrus chilensis* (D. Don) Pic. Ser. *et* Bizzarri. These authors verified the feasibility of applying the grafting technique for the preservation of genetic diversity, as the populations of this species are under eminent threat.

The propagation of *S. toromiro*, as proposed by Maunder *et al.* (2000), involves only propagation through seeds, stakes and micropropagation. Studies addressing micropropagation have been performed by Iturriaga *et al.* (1994) and Jordan *et al.* (2001) and agree regarding the recalcitrance of this species and the difficulty of its propagation.

According to the results obtained in this study, graft generation at an operational scale established in an arboretum or clonal bank represents a strategy to be considered for seed collection through controlled or open pollination. Nursery techniques and further restoration may achieve better adaptation for certain sectors that were previously selected on Easter Island, once as sufficient seeds become available.

Alternatively, the grafts obtained in this study may be used as mother plants to provide vegetative material for studies on macro- and micropropagation. Due to the species' conservation status, care must be taken both to plant this material in a suitable area for development and in its monitoring and management.

Ideally, establishment on Easter Island may seem logical, but a habitat as extremely modified as this island is not safe, particulary given the historical failure of reintroductions of this species. Therefore, introduction in an alternative area established on the continent is advisable, in protected areas between the regions of Valparaíso and Bío Bío, with a coastal influence. These areas are more appropriate because the edaphoclimatic conditions of the zone will allow the specimens to survive, resulting in the induction of floral buds in the medium term. In fact, more than 800 grafts have been established in the Reserva Nacional Lago Peñuelas in the administrative region of Valparaíso (33°10´52" S; 71°29'35" W) as a result of an agreement between the company Forestal Mininco S.A. and the Corporación Nacional Forestal (CONAF). The planting density of the specimens is 0.5 x 4 m, covering an area of 0.45 ha. The recorded plant survival to date is approximately 95%.

Alternative areas should be selected in arboretums in the previously mentioned regions to fulfill the role of protecting the germplasm of species with conservation problems, thus guaranteeing the availability of synthesized material. A further stage will involve the establishment of the available lines as a strategy for augmenting the genetic pool of this species. Once a greater number of offspring are obtained, massive reintroductions could be proposed for Easter Island itself.

It is also important to note the intrinsic roles of genetics and conservation. According to Mills and Soulé, as cited by Allendorf and Luikart (2007), the perspective of these disciplines some decades ago was that "many conservationists had ignored genetics and that many geneticists had ignored the catastrophe of biodiversity". This is reflected in the low success of conservation programs targeting plants that are classified as extinct or endangered.

Propagating *S. toromiro* using the technique reported here appears to be a solution for the *ex situ* conservation of this species based on the good propagation results that were experimentally demonstrated in this study. Additionally, using grafting propagation to produce seeds is fully justified by the low number of specimens of the species available in both Chile and in the botanic gardens in Europe.

It was therefore validated in this study that grafting is an efficient tool for vegetative propagation that fulfills an important role in biodiversity conservation, contributing to the rescue of species with conservation problems in the medium term, the obtained grafts will provide seeds, which through additional nursery techniques, will provide plants for further experiments to obtain recruits for favorable sectors for development on Easter Island.

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Resumen

J. Espejo, M. Baeza, E. Ruiz, F. Mora, M. Gomez y G. Montenegro. 2013. Masificación de *Sophora toromiro*, mediante injerto inter específico como apoyo a la conservación de la especie. Cien. Inv. Agr. 40(1):213-221. El presente documento entrega antecedentes de la técnica de injerto por hendidura con la especie *Sophora toromiro* (Phil.) Skottsb., orientada a su propagación y contribuir a la conservación de su base genética en el mediano y largo plazo. La captura de germoplasma se realizó por medio de injertos inter específicos, utilizando *Sophora cassioides* (Phil*.)* Sparre como porta injerto, con dos variantes de sellado de injerto de hendidura. La sobrevivencia de 301 injertos de *S. toromiro* de la línea Titze, a los diez meses, es de un 64 y 95%, según el tipo de sellado utilizado. Un segundo experimento, para contrastar origen de material, entrega que la procedencia Goteborg alcanza valores cercanos al 63% de efectividad versus un 58% del Jardín Botánico Nacional de Viña del Mar (Chile). Este trabajo entrega un nuevo enfoque para la recuperación de *S. toromiro,* y otras especies leñosas que están catalogadas en peligro de extinción o bien amenazadas, mediante la técnica de injerto de hendidura.

Palabras clave: Conservación, injerto, *Sophora toromiro, S. cassioides*.

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