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Research article

Clonación de *Pinus patula* Schiede ex Schldl. & Cham. por injerto: efecto de la fenología y tipo de púa

Cloning of *Pinus patula* Schiede ex Schldl. & Cham. by grafting: effect of phenology and type of scion

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Abstract

Grafts allow the cloning of select genotypes, but the lack of sprouting in conifers is common due to intrinsic and extrinsic causes of the grafting process. The objective was to evaluate the sprouting and survival of side veneer grafts of *Pinus patula* with scions in three phenological stages (dormancy, beginning of growth and full growth), two types of scions (terminal buds and basal scions) and their interaction, in addition to determining the average sprouting time due to the effect of the treatments. A completely randomized experimental design was used with a 3×2 factorial arrangement with 16 replications. The evaluation was biweekly until 90 days after grafting. Analysis of variance was performed to detect differences between treatments and the accelerated failure time and Weibull hazard ratio models were adjusted to estimate the average graft sprouting time and probability of death, respectively. No significant differences were found ($p<0.05$) between treatments. In all combinations there was sprouting (79.2 to 100 %) and survival varied between 58 and 83 %. 100 % of sprouting was obtained with terminal buds in dormancy and basal scions in full growth, while the highest survival (83 %) was accomplished with basal scions at the beginning of growth and terminal buds in full growth. The shortest average sprouting time was achieved when grafting basal scions (15 days). *Pinus patula* can be successfully cloned at any phenological stage and with the use of both types of scions evaluated.

Key words: Phenological stages, side veneer graft, sprouting, asexual propagation, basal scions, scions with terminal buds.

Resumen

Los injertos permiten clonar genotipos selectos, pero es común la falta de prendimiento en coníferas por causas intrínsecas y extrínsecas del proceso de injertado. El objetivo del presente estudio fue evaluar el prendimiento y la supervivencia de injertos de enchapado lateral de *Pinus patula* con púas en tres estadios fenológicos (latencia, inicio del crecimiento y pleno crecimiento), dos tipos de púas (yemas terminales y púas basales) y su interacción, además de determinar el tiempo promedio de prendimiento por efecto de los tratamientos. Se usó un diseño

experimental completamente al azar con arreglo factorial 3×2 con 16 repeticiones. La evaluación fue quincenal hasta los 90 días después del injertado. Se realizaron análisis de varianza para detectar diferencias entre tratamientos y se ajustaron los modelos de tiempo de fallo acelerado y razón de riesgo de *Weibull* para estimar el tiempo promedio de prendimiento y la probabilidad de muerte de los injertos, respectivamente. No se registraron diferencias significativas ($p < 0.05$) entre tratamientos. En todas las combinaciones hubo prendimiento (de 79.2 a 100 %) y la supervivencia varió entre 58 y 83 %. Se obtuvo 100 % de prendimiento con yemas terminales en latencia y púas basales en pleno crecimiento, mientras que la mayor supervivencia (83 %) se presentó con púas basales al inicio del crecimiento y yemas terminales en pleno crecimiento. El menor tiempo promedio de prendimiento se logró con púas basales (15 días). *Pinus patula* se puede clonar exitosamente en cualquier estadio fenológico y con el uso de ambos tipos de púas evaluadas.

Palabras clave: Estadios fenológicos, injerto de enchapado lateral, prendimiento, propagación asexual, púas basales, púas con yemas terminales.

Introduction

Asexual propagation allows superior genotypes to be cloned (Darikova *et al.*, 2011). It is divided into macropropagation (grafting, rooting of cuttings and aerial or underground layering) and micropropagation (*in vitro* tissue culture) (Hartmann *et al.*, 2010). The graft consists of joining a bud, shoot or branch (scion or twig) of an adult tree on the stem or root (rootstock) of a seedling or sapling (not older than two years, preferably), which evolve physiologically and anatomically to form a new plant (González-Jiménez *et al.*, 2022). Its use is extensive in fruit trees and vegetables (Pérez-Luna *et al.*, 2019).

In conifers, this propagation alternative has increased in the last 50 years to establish asexual seed orchards, in which the aim is to obtain genetically improved seed in abundant quantities by crossing superior trees (Yuan *et al.*, 2016; Stewart *et al.*, 2017). However, the effect of some factors that influence graft attachment and survival is still unknown in detail, particularly in Mexican pine species (Barrera-Ramírez *et al.*, 2021).

In most experiments carried out in pine trees, survival of less than 50 % has been recorded (Muñoz *et al.*, 2013; Pérez-Luna *et al.*, 2019; Barrera-Ramírez *et al.*, 2021). Therefore, the assessment of some variations in grafting techniques will favor the reduction of mortality rates. In Mexico, *Pinus patula* Schiede ex Schltdl. & Cham. (González-Jiménez *et al.*, 2022), *P. pseudostrobus* Lindl. (Barrera-Ramírez *et al.*, 2021) and *P. engelmannii* Carrière (Pérez-Luna *et al.*, 2021) are the pine species in which mostly grafting has been performed, and their survival percentages vary from 25 to 90 %.

The main techniques used to graft conifers are top cleft and side veneer, although the latter has been used to a lesser extent (Muñoz *et al.*, 2013). The vigor and quality of the scions influences the success of a graft, which is why scions with terminal buds from the last growth cycle are usually used (Pérez-Luna *et al.*, 2021). However, it is difficult to have material to perform a large number of grafts per tree, when the terminal buds that come from an orchard (donor tree) are scarce or from young donors. Therefore, in *Pinus greggii* Engelm. ex Parl. var. *australis* Donahue, Jeffrey K. & López A., R., the use of terminal buds and basal scions or twigs (section of the base of a shoot from the last growth cycle) was tested with a survival greater than 90 % in both cases (Castro-Garibay *et al.*, 2022). On the other hand, survival models have been little used to evaluate grafting success (Pérez-Luna *et al.*, 2019). A useful model is the Weibull accelerated failure time model since it allows predicting the average time in which an event occurs (Pérez-Luna *et al.*, 2021).

Pinus patula is the species of greatest economic and ecological importance in south-central Mexico, due to its wood quality and rapid knot-free growth (Leibing *et al.*, 2013). This has made it one of the conifers most used in genetic improvement programs in several countries in the southern hemisphere and Mexico (Muñoz-Gutiérrez *et al.*, 2017).

The objective of the present study was to evaluate the effect of the phenological condition of the grafted buds (dormancy, beginning of growth and full growth) and

the use of two types of scions (terminal and basal buds) on the attachment, survival and risk of death of lateral veneer grafts of *Pinus patula*. The hypothesis was that phenology, the type of scion used and/or the interaction between the two have a significant effect on graft sprouting, survival and risk of death.

Materials and Methods

Rootstock production

The rootstocks were obtained from seed collected from 20 trees selected for their outstanding size in height (26 to 34 m) and diameter (35 to 40 cm), located in stands of the *ejido Peñuelas Pueblo Nuevo, Chignahuapan, state of Puebla* (19°57'21 " N and 98°06'40" W, 2 650 m). The plant was produced in the nursery of the *Programa del Posgrado en Ciencias Forestales del Colegio de Postgraduados* (Graduate Program in Forest Sciences of the Graduate Studies College), at *Texcoco, Mexico* (19°27'37" N and 98°54'23" W, 2 240 m). Sowing was done in individual plastic containers with a 310 cm³ capacity, with a substrate composed of peat moss, perlite and vermiculite (2:1:1). One year after sowing, the transplant was carried out into 19×22 cm (diameter×height) black plastic bags of 6 L capacity, with a substrate based on a mixture of composted pine bark, peat, perlite and pine sawdust (4:2:2:2).

Collection and handling of scions

On April 29th, 2023, shoots from the last growth cycle of 12 superior trees were collected, based on their growth and conformation, located in *Chignahuapan* and *Ixtacamaxtitlán, Puebla*. The selected trees had dominant height (18 to 26 m) and diameter (19 to 25 cm), straight stem, branch insertion at an angle close to 90° and good natural pruning. In each provenance, six trees were selected: two with dormant buds, two at the beginning of bud growth and two with buds in full growth. The phenological stages were defined based on the growth characteristics of the buds; scions in dormancy were those in which no bud elongation was observed (Figure 1A), while growing scions showed initial elongation (start of growth) (Figure 1B) and bud development (full growth) (Figure 1C).



A = Dormancy; B = Beginning of growth; C = Full growth.

Figure 1. Phenological stage of scions.

To keep the scions moist and prevent dehydration, transportation was carried out in a plastic cooler with frozen hydrogel bags inside, thus avoiding direct contact with the twigs. The scions were subjected to the following handling before grafting: (a) Removal of needles, (b) Disinfection with water, liquid soap and 2 % chlorine, (c) Soaking in a solution based on water and Captán® 50PH fungicide, at a rate of 10 g L⁻¹ for 15 minutes, (d) Rinse with distilled water, and (e) Store in a cooler around 15 minutes before grafting.

Establishment of the test and graft procedure

The grafts were carried out on April 30th, 2023 in the nursery of the *Posgrado en Ciencias Forestales del Colegio de Postgraduados* (Graduate Program in Forest Sciences of the Graduate Studies College) in the *Montecillo* Campus, *Texcoco*, Mexico. The rootstocks were 18 months old and their average height and diameter at the root neck were 70 and 1.5 cm, respectively. The side veneer graft technique described by Pérez-Luna *et al.* (2019) was followed. The scion was prepared by two parallel tangential cuts of 5 and 1 cm in length each (Figure 2A). A 4 cm long tangential cut was made in the pattern with a slight 1 cm deep indentation (Figure 2B) to achieve an adequate fit of the graft, which was fixed with 4 mm caliber glass rubber. Two types of twigs were used for grafting: scions with terminal bud (Figure 2C) and basal scions (section of the base of the twig collected in the field, generally discarded in grafting activities) (Figure 2D). The graft was covered with a 0.75 L transparent plastic bag, with disinfectant based on quaternary ammonium salts (10 mL L⁻¹ of water) applied by atomization to generate a microclimate of high relative

humidity (Figure 2E), which was covered with a Kraft paper bag to protect the graft from solar radiation and prevent dehydration (Figure 2F).



A = Cut in the scion; B = Cut in the rootstock; C = Scion graft with terminal bud;
 D = Basal scion graft; E = Microclimate with moistened plastic bag; F = Grafts covered with a Kraft paper bag.

Figure 2. Grafting process.

The paper bag was removed 15 days after grafting, the microclimate was eliminated after 30 days and at 60 days the release (pruning of the central stem) of the graft began. The grafts were maintained in a shade mesh condition with 60 % light retention and were watered three times a week with water added with Peters® triple 20 (N-P-K) water-soluble fertilizer at a dose of 5 g L⁻¹. The maximum, minimum and average temperature observed during the evaluation period was 28.4, 2.7 and 16.7 °C, respectively. The relative humidity of the site where the grafts were kept fluctuated between 48 and 66 %.

Treatments and experimental design

The treatments evaluated were based on the phenology of the scions of the donor tree (dormancy, initiation of growth and full growth) and the type of grafted shoot (scion with bud and basal), in addition to their interaction. In total, six treatments were evaluated: three phenological stages \times two types of scion. For each phenological stage, 32 grafts were made, half were with terminal buds and the other half with basal scions. The experiment was distributed in a completely randomized design with a 3×2 factorial arrangement with 16 replications. In total 96 grafts were evaluated. Sprouting (elongation and generation of new needles) and survival (graft with turgid tissue) were evaluated every 15 days until 90 days after grafting with dichotomous variables: graft with sprouting (1), without sprouting (0), and graft alive (1) and dead (0). The statistical model used was:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \epsilon_{ijk} \quad (1)$$

Where:

Y_{ijk} = Value of the response variable of the k^{th} replication of the i^{th} level of A and the j^{th} level of B

μ = General mean

A_i = Effect of the i^{th} level of A factor (phenological stage)

B_j = Effect of the j^{th} level of B factor (scion type)

AB_{ij} = Effect of the interaction of the i^{th} level of A and j^{th} level of B

ϵ_{ijk} = Experimental error

Statistical analysis

With the data that were obtained, an analysis of variance (ANOVA) for binomial distribution was carried out with the generalized linear mixed procedure (GLIMMIX) of SAS version 9.4 (SAS Institute, 2013) to detect statistical differences ($p < 0.05$) between treatments on attachment and survival. The Weibull accelerated failure time model (Equation 2) was fitted to determine whether the phenology of the scion donor trees and scion type had a significant effect on the average time to grafting and mortality of the grafts.

$$\ln(T) = \alpha + \beta x_i + \sigma \epsilon \quad (2)$$

Where:

$\ln(T)$ = Natural logarithm of accelerated failure time (sprouting and mortality)

α = Model shape parameter

βx_i = Estimator attributed to the i^{th} treatment

$\sigma \epsilon$ = Scale parameter attributed to model error

On the other hand, the Weibull hazard ratio (Equation 3) and the probability of death (Equation 4) were calculated with the equations described by Pérez-Luna *et al.* (2021).

$$HR_w = (\exp^{-\delta})^{\lambda-1} \quad (3)$$

$$\text{Probability of death} H = (HR_w - 1)(100) \quad (4)$$

Where:

HR_w = Weibull hazard ratio (increase or decrease in the risk of death depending on an independent variable), which can take values between -1 and 1

\exp = Euler number (approximately 2.7182)

δ = Coefficient indicating the effect of an independent variable (x_i)

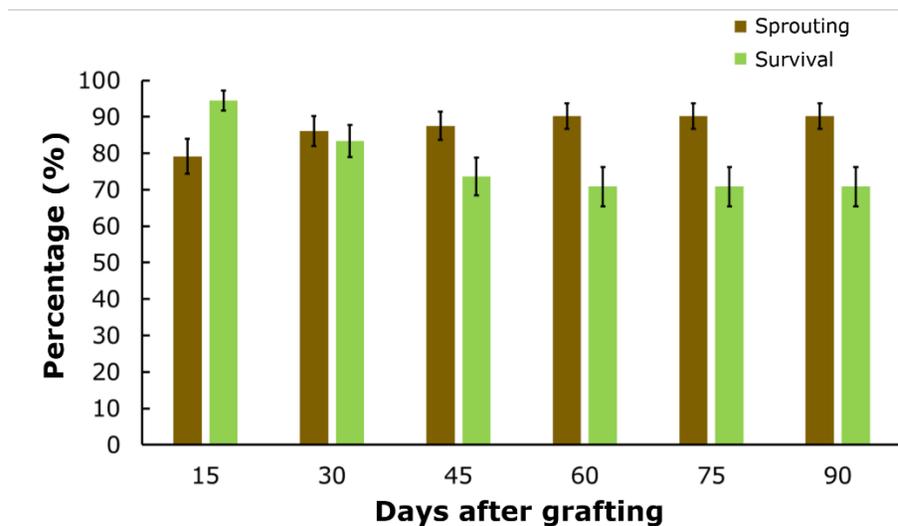
λ = HR_w model shape parameter

To fit the model, we worked with dichotomous and polytomous variables: attachment (graft without sprouting=0 and with sprouting=1), survival (live graft=0 and dead=1), phenology of the donor tree (dormancy=1, beginning of growth=2 and full growth=3) and type of scion (scion with terminal bud=1 and basal scion=2). The Weibull model uses censoring variables (value equal to 0) to detect individuals who did not experience the effects to be evaluated (in this case, graft without sprouting and live graft). Fittings of the accelerated failure time and Weibull hazard ratio models were performed using the LIFETEST and PHREG procedures of SAS version 9.4 (SAS Institute, 2013).

Results and Discussion

Sprouting and survival

There were no significant differences ($p < 0.05$) between the evaluation dates for grafting and average graft survival (Figure 3).



Vertical lines represent the standard error.

Figure 3. Average graft sprouting and survival by evaluation period (days after grafting).

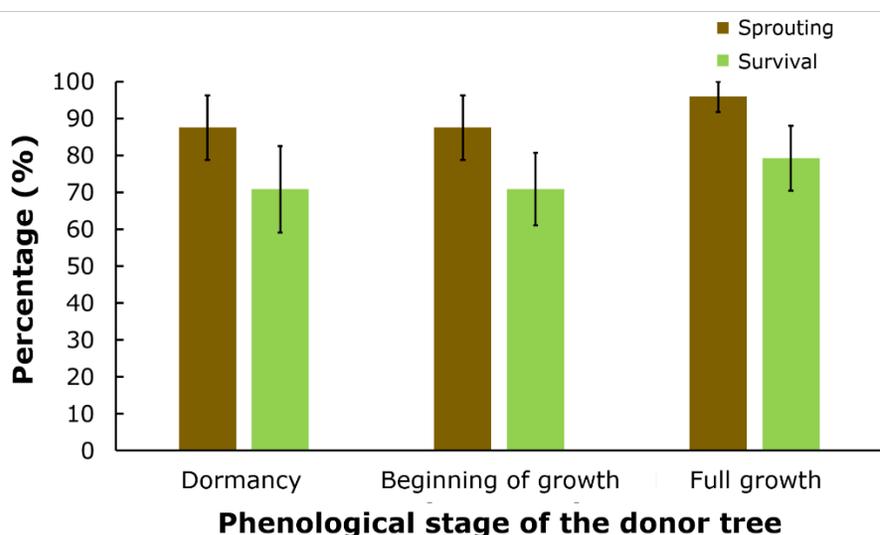
A high sprouting was observed from 15 days after grafting (79.2 %) until increasing to 90.3 % at 60 days, and then remaining stable. As expected when performing grafts, a decrease in survival was identified in each assessment, which varied from 94.4 to 70.8 % from the initial to the final evaluation. The fact that there was greater sprouting than survival is explained by the fact that not all grafts that show signs of “compatibility” can survive over time, either due to poor union of the vascular cambium, the death of the rootstock and/or of the scion due to stress, pest

attack or poor nutrition (Barrera-Ramírez *et al.*, 2021). In this regard, no apparent causes of mortality were detected, so it is presumed that the death of the sprouted grafts could have been due to early incompatibility.

Historically, the top cleft technique has been the most used to clone coniferous species (particularly pines); results have been successful in some tests, such as *Pinus sylvestris* L. with a survival rate of 85 % (Almqvist, 2013), *P. engelmannii* with 80 % (Pérez-Luna *et al.*, 2021) and *P. greggii* var. *australis* with 89 % (Castro-Garibay *et al.*, 2022). However, for *P. rzedowskii* Madrigal & Caball. Del., it seems to be more suitable to make side veneer grafts (sprouting greater than 80 %) (Solorio-Barragán *et al.*, 2021). Likewise, other studies on *P. patula* indicate that side veneer is an efficient methodology for the vegetative propagation of adult trees of the species (González-Jiménez *et al.*, 2022; González-Jiménez *et al.*, 2023).

The age and quality of the rootstock is a factor that usually influences the success of grafts of the *Pinus* L. genus; therefore, it is recommended to use rootstocks no older than 15 months when grafting *P. patula* and other phylogenetically close species (Castro-Garibay *et al.*, 2022). However, the results obtained in the present investigation and those reported by González-Jiménez *et al.* (2022) suggest that *P. patula* can be successfully grafted using rootstocks up to 18 months old. This is useful as it makes it easier to graft scions from adult trees, which reduces the incompatibility that a graft could present due to the difference in growth between the vegetative organs used, since scions from adult trees usually grow faster in diameter than young rootstocks (Pérez-Luna *et al.*, 2019).

No statistical differences were detected ($p < 0.05$) in the sprouting and survival due to the phenological stage of the donor trees at 90 days after grafting (Figure 4). This despite the fact that there was a difference of 11.3 % in sprouting (98.8 vs. 87.5) and 8.3 % in survival (79.1 vs. 70.8) between the extreme values.



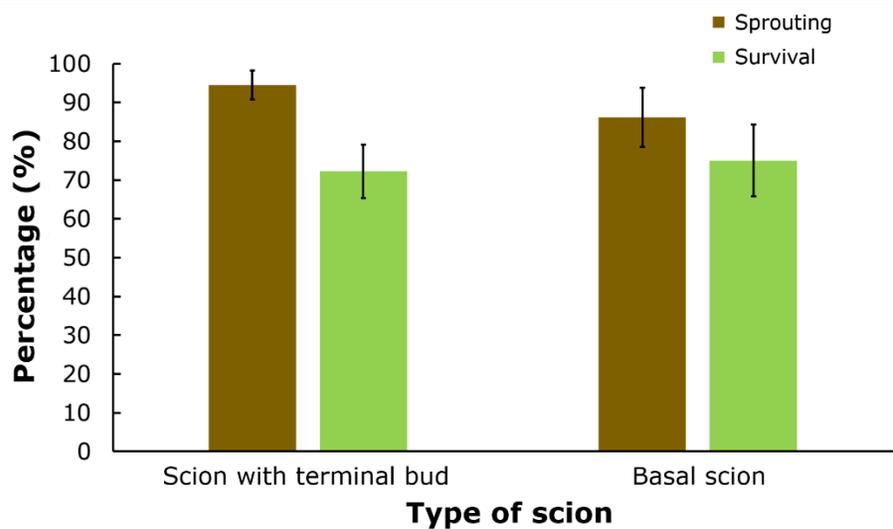
Vertical lines represent the standard error.

Figure 4. Graft sprouting and survival by phenological stage of the donor tree 90 days after grafting.

In *P. engelmannii*, greater survival was recorded when using scions in dormancy (26.6 %) compared to the beginning of growth (18.3 %) (Pérez-Luna *et al.*, 2019). This indicates that *P. patula* is suitable for grafting at any phenological stage, and this could be due to the rapid growth of the species and the number of growth cycles it has annually (Leibing *et al.*, 2013).

The results obtained are similar to those of González-Jiménez *et al.* (2023) for the same species, as they report sprouting and survival of 74.7 and 50 %, respectively, when performing side veneer grafts with scions in full growth. This is opposite to what was proposed by Barrera-Ramírez *et al.* (2021) and Pérez-Luna *et al.* (2021), who recommend grafting with dormant twigs in *P. pseudostrobus* and *P. engelmannii*. However, it must be taken into account that the behavior of the grafts may be different depending on each species.

The type of scion did not influence the response variables 90 days after grafting ($p < 0.05$); the sprouting values varied from 94.4 to 86.1 %, while the survival values fluctuated between 75 and 72.2 % (Figure 5).

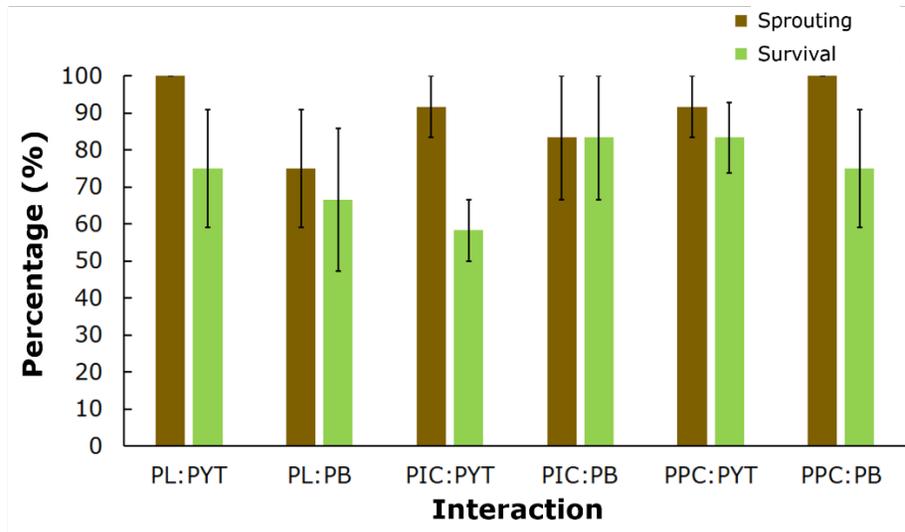


Vertical lines represent the standard error.

Figure 5. Graft sprouting and survival by type of scion 90 days after grafting.

It would be expected that in grafts with terminal buds there would be greater sprouting given the greater photosynthetic function and carbohydrate production that this type of scions have (González-Jiménez *et al.*, 2023), a situation that was observed despite not being statistically different from the use of basal scions. However, this aspect could also affect survival due to the greater amount of resources (water and nutrients) that they require for their correct functionality, which could make them vulnerable to external factors such as pests, diseases and water stress (Marmolejo *et al.*, 2020). In this regard, the use of basal scions can be efficient for grafting *P. patula* from the good regrowth ability of the species (Aparicio-Rentería *et al.*, 2014).

The interactions did not show a significant effect ($p=0.05$) with respect to attachment and survival 90 days after grafting (Figure 6).



PL = Scions in dormancy; PIC = Scions at the beginning of growth; PPC = Scions in full growth; PYT = Terminal bud scions; PB = Basal scions. Vertical lines represent the standard error.

Figure 6. Graft sprouting and survival from the effect of the phenological stage x scion type interaction 90 days after grafting.

The highest sprouting (100 %) was found in grafts with terminal buds from dormant donor trees and basal scions from trees in full growth, while the lowest (75 %) occurred in the basal scions from dormant trees. Regarding survival, the highest value (83 %) was reached when grafting basal scions and terminal buds of trees at the beginning of their growth and in full growth, respectively. In contrast, individuals grafted with terminal buds of trees at the beginning of their vegetative phenology showed the lowest average survival with 58 %. The percentage of basal scion grafts from trees in full growth is similar to those reported by Castro-Garibay *et al.* (2022), who observed 98 % sprouting in *Pinus greggii* var. *australis* by

grafting basal segments of fully growing shoots. On the other hand, high survival percentages have been obtained in other species when using scions with terminal buds from dormant donor trees, such is the case of *P. mugo* Turra (Świerczyński *et al.*, 2020) and *P. engelmannii* (Pérez-Luna *et al.*, 2021), both with 80 %.

Sprouting time and risk of death

A significant effect ($p < 0.05$) of the type of scion on the average sprouting time and the risk of death of the grafts was identified (Table 1). A significant effect of the phenological stage of the donor trees on the evaluated variables was not confirmed.

Table 1. Adjustment parameters of the accelerated failure time model and Weibull hazard ratio in the sprouting of *Pinus patula* Schiede ex Schltdl. & Cham. grafts due to the effect of the type of scion.

Accelerated failure time			
Parameter	Estimator	 z 	p value
Shape (σ)	2.6659	9.99	<0.0001
"Kind of scion" variable coefficient (β)	0.4373	2.56	0.01
Scale (σ)	-0.3757	-4.30	<0.0001
Weibull hazard ratio			
Parameter	Estimator	p value	
Shape parameter (λ)	0.0206	< 0.0001	
"Kind of scion" variable coefficient (δ)	1.4559	0.01	

The average sprouting time of the grafts with terminal buds and basal scions was 15 and 23 days after grafting, respectively. The value of the hazard ratio when

$x=1$ (scion with terminal bud) was 0.53. This indicates that the probability of death $[(0.53-1) \times 100]$ decreases by 47 % when using this type of scion. The hazard ratio with the use of basal scions ($x=2$) was 0.28, with a decrease in the probability of graft death of 72 % $[(0.28-1) \times 100]$. Castro-Garibay *et al.* (2022) tested the use of scions with terminal buds and basal scions when making terminal fissure grafts of *P. greggii* var. *australis* and obtained satisfactory results, with a survival of 83 and 87 %, respectively. However, in this study the average sprouting time was not specified.

Few investigations on *Pinus* grafts have made use of survival models to determine factors that reduce or increase the risk of death. In *P. engelmannii*, the risk of mortality of the grafts was evaluated due to the phenology of the donor trees (Pérez-Luna *et al.*, 2021), in which a decrease in the risk of death was determined when using scions in dormancy. However, there is no record of its use in *P. patula*. This makes survival models a useful and underutilized tool in similar research.

Conclusions

Pinus patula can be successfully cloned by side veneer grafts with terminal bud spikes and basal scions from donor trees at any phenological stage of their growth, thereby maximizing the material available for grafting activities. Side grafts of scions with terminal bud require less time to show sprouting, although basal scions apparently allow slightly better survival results to be obtained.

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Conflict of interest

The authors declare no conflict of interest.

Contribution by author

Alberto Pérez Luna: execution and supervision of the research study, data capture, statistical analysis and interpretation of results and writing of the manuscript; Javier López Upton: methodological design, execution and supervision of the research study, statistical analysis and interpretation of results and review of the manuscript; José Ángel Prieto Ruíz and Rubén Barrera Ramírez: statistical analysis, interpretation of results and review of the manuscript.

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