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Research article

Fenología de estructuras reproductivas de *Pseudotsuga menziesii* (Mirb.) Franco

Phenology of reproductive structures of *Pseudotsuga menziesii* (Mirb.) Franco

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Abstract

Determining the beginning, the end, and the time length of each reproductive stage in ovule receptivity as well as in pollen dispersal allows to generate information for a genetic improvement program. The general goal was to determine the period for the receptivity of female strobili and pollen dispersal on a young tree plantation of *Pseudotsuga menziesii* treated with gibberellic acid ($GA_{4/7}$ and GA_3) to promote flowering. Twelve trees with reproductive structures were monitored; three branches with female strobili and three branches with male structures were marked in each tree. The initial and end dates and time length of each of the phenological stages of flowering were registered and determined with the SYNCHRO macro using SAS software. The receptivity period of female strobili lasted on average 14 days, while pollen dispersal lasted between five and six days. The female receptivity periods and pollen dispersal showed high synchronization (P=0.60 and 0.64), while the correlation coefficients between phenological events are high and positive (r=0.87), in addition to being significant values ($p \leq 0.001$). Thus, the phenological events of pollen dispersal and female receptivity are highly synchronized and are not independent, so there is the probability of crossing between the trees.

Key words: Gibberellic acid, pollen dispersal, floral induction, seed management, female receptiveness, phenological synchronization.

Resumen

Conocer el inicio, el fin y la duración de la receptividad femenina y de la dispersión de polen permite generar información para el establecimiento de programas para la producción y manejo de semillas, así como de mejoramiento genético. El objetivo de este estudio fue determinar los periodos de receptividad de los estróbilos femeninos y la dispersión de polen en una plantación de árboles jóvenes de *Pseudotsuga menziesii* que se han tratado con ácido giberélico ($AG_{4/7}$ y AG_3) para promover la floración temprana. Se dio seguimiento a 12 árboles con estructuras reproductivas femeninas y masculinas. Se definieron las fechas de inicio, fin y duración de cada una de las etapas fenológicas de la floración con el macro *SYNCHRO* de SAS. El periodo de receptividad de las estructuras femeninas fue, en promedio, de 14 días, mientras que la dispersión de polen duró entre cinco y seis días. Las etapas de receptividad femenina y la dispersión de polen presentaron alta sincronización (*PO*=0.60 y 0.64), los coeficientes de correlación entre los eventos fenológicos fueron altos, positivos (r=0.87) y significativos ($p \leq 0.001$). Se concluye que la dispersión de polen y la receptividad femenina están sincronizados y no son independientes, por lo que existe la probabilidad de cruzamiento entre los árboles.

Palabras clave: Ácido giberélico, dispersión de polen, inducción floral, manejo de semillas, receptividad femenina, sincronización fenológica.

Introduction

The *Pseudotsuga* genus (Pinaceae) known as fir or Douglas fir is distributed in Western North America and Southeast Asia (Farjon, 1990). *Pseudotsuga menziesii* (Mirb.) Franco develops in varied environments and its geographical distribution is wide, forming extensive natural forests in the Western United States of America and Canada (Hermann and Lavender, 1999).

In Mexico, the natural distribution of *P. menziesii* is geographically extensive, although it is found in small populations or isolated patches along the *Sierra Madre Occidental* and in the Northern part of the *Sierra Madre Oriental* and the *Sierra de Querétaro* (Rzedowski, 1978). In addition, it forms small stands along the Neovolcanic Axis in the center of the country (Villagómez and Bello, 2015), in *Tlaxcala* and *Puebla* (Domínguez *et al.*, 2004). Also, two populations are cited in the state of *Oaxaca*: one in *Santa Catarina Ixtepeji* (Debreczy and Rácz, 1995) and another in the *Sierra Madre del Sur*, both representing the Southern natural limit of the species (Del Castillo *et al.*, 2004).

In the national territory, *P. menziesii* is highly appreciated for its potential as a Christmas tree. Therefore, germplasm is required to establish commercial forest plantations (CFP) and at the same time to conserve the species in its natural populations.

Currently, planters demand a large quantity of seeds for Christmas tree CFPs, mainly in the central area of the country (*Puebla*, State of Mexico and *Tlaxcala*). Some alternatives to solve this supply problem consist of having a better knowledge of the factors that regulate flowering and seed production in trees. To this end, there are cultivation practices and treatments that have been used successfully to accelerate the production of cones and seeds in juvenile trees, based on practices such as trunk girdling and the application of growth regulators, root pruning, stress temperature and fertilization (Ross and Bower, 1989; Cherry *et al.*, 2007; Muñoz-Gutiérrez *et al.*, 2010).

Floral induction through the addition of growth regulators that accelerate cone and seed production in a wide range of conifers is well known, particularly with gibberellic acid (GA). The combination of GA_4 and GA_7 ($GA_{4/7}$) is the most effective and is used in the induction of reproductive structures in different conifer species (Ross, 1983; Greenwood, 1987; Owens *et al.*, 2001; Aderkas *et al.*, 2004; Cherry *et al.*, 2007). However, these treatments, in addition to promoting the formation of reproductive structures, may modify the periods of pollen receptivity and dispersal (Vargas-Hernández and Vargas-Abonce, 2016), a modification that can reduce or eliminate the contribution of some individuals in the seed production (Muñoz-Gutiérrez *et al.*, 2010). The above is due to the lack of phenological synchrony between individuals from the same plantation, which increases the probability of inbreeding, and therefore the number of wasted seeds (Burczyk and Chalupka, 1997).

Although there are already precedents in Mexico on floral induction work in *P. menziesii* (Muñoz-Gutiérrez *et al*., 2010), there are no studies on its reproductive phenology in natural populations or in Christmas tree plantations with young trees treated to stimulate early flowering. Therefore, the general objective of the present work was to determine the periods of receptivity of female strobili and pollen dispersal in a plantation of young *P. menziesii* trees that were treated with gibberellic acid ($GA_{4/7}$) to induce the formation of reproductive structures. Two groups of trees with different levels of response to the formation of female and male reproductive structures were used; it was proposed to analyze, in particular, the differences between groups of trees in terms of the beginning, end and duration of the receptivity of the female strobili and pollen dispersal, in addition to their relationship with the accumulation of degree-days, and determine the degree of phenological overlap between individuals in the same plantation. The hypothesis raised is that the synchrony between female receptivity and pollen dispersal is different between two groups of young *P. menziesii* trees due to the differential response to the formation of male and female reproductive structures.

Materials and Methods

Location of the plantation

The *P. menziesii* Christmas tree plantation is located in the *Bosque Esmeralda* Ecotourism Park, which belongs to the *Emiliano Zapata ejido*, *Amecameca* municipality, State of Mexico, at the coordinates 19°07’14” N and -98°44’06” W, at 2 640 masl (SBE, 2020).

Floral induction assays

In a section of the park's *P. menziesii* plantations, 12 young trees aged between seven and eight years, with a height of 1.60 to 2.60 m and trunk diameter of 5.20 to 9.50 cm, all in full sunlight condition, were selected. Two treatments were applied to the trees to induce flowering:

Treatment 1: Girdling on the trunk and application of a single dose of gibberellic acid $GA_{4/7}$ (Green Import Solutions®, Japan) (2.55 mg cm^{-2} of basimetric area) in a single event, but on different dates between the months of March to April 2021 with seven-days intervals.

Treatment 2: Girdling on the trunk and a single application (March 8th, 2021) of $GA_{4/7}$ (Green Import Solutions®, Japan) (2.55 mg cm^{-2} of basimetric area). Girdling is a partial blockage of the phloem to promote greater availability of carbohydrates and other growth-promoting substances (Ross and Bower, 1989), which consisted of making two transverse cuts to the trunk in the shape of a crescent with a saw, the first cut was made 50 cm from the ground and the second at a distance of 1.5 times the diameter of the trunk, after the first cut (Muñoz-Gutiérrez *et al.*, 2010).

Phenology of reproductive structures

Monitoring was carried out on 12 trees with the formation of female and male reproductive structures during the months of February and March 2022, regardless of the type of treatment. For each tree, three branches located between the middle and upper part of the crown were marked, since it is the section with the greatest presence of female reproductive structures, and three branches for monitoring the male structures, which were located between the middle and lower part of the crown, where the male strobili mainly develop.

Observations of phenological progress were made with a frequency of four to five days from the moment the reproductive structures were visualized in the buds, until the female structures were no longer receptive, and when pollen dispersal in the male strobili ended.

To determine the phenological stages, the methodology described by Webber and Painter (1996) was followed. For the female strobili, five stages were defined: (E1) The female reproductive structures are distinguished, not yet emerging, in the bud, (E2) Elongation of the strobile, burst bud and the bracts of the reproductive structure are visible, (E3) 30 to 40 % of the bracts are exposed, the female structure is already receptive, (E4) Maximum receptivity, and (E5) Formation and elongation of the female strobile (Figure 1).

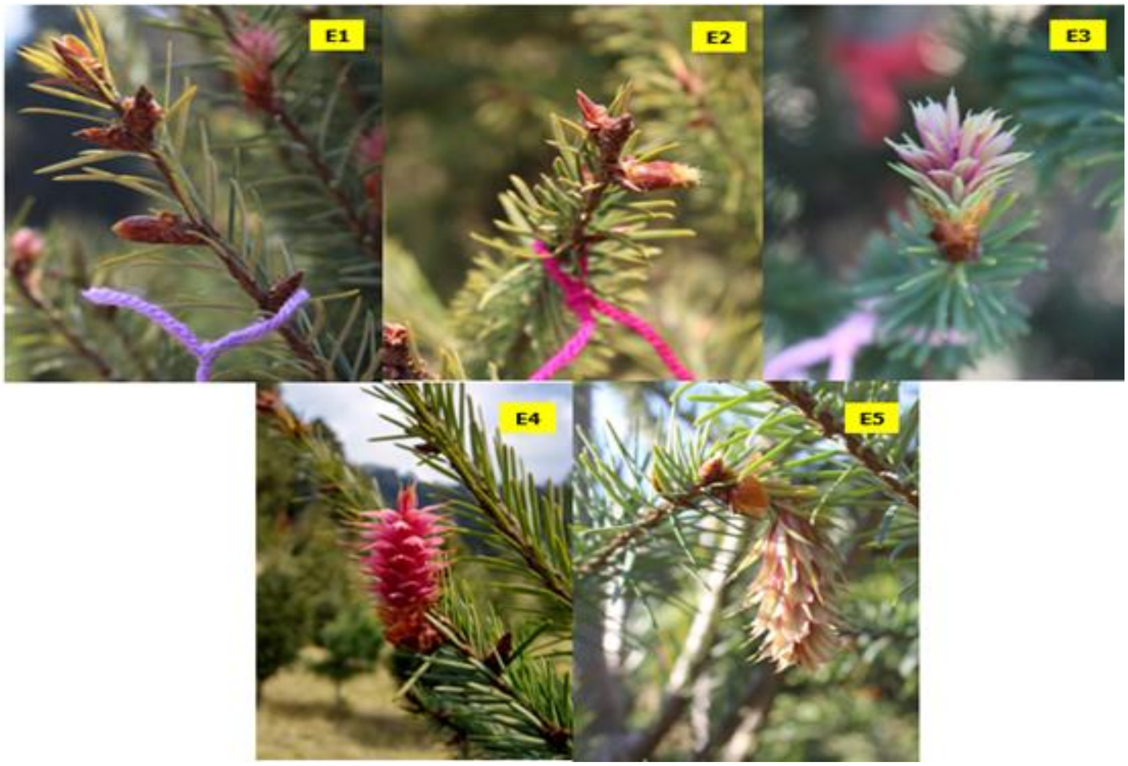

Figure 1. Phenological stages of female strobili of *Pseudotsuga menziesii* (Mirb.) Franco.

For male strobili, five stages were considered too: (E1) Male reproductive structures are distinguished, not yet emerging in the buds, (E2) The buds swell and the pollen sacs are visible, (E3) Elongation of the strobile, breaks the scales of the bud, (E4) The strobile reaches maximum size and maturation, a phase in which the pollen is dispersed, and (E5) Dispersion ends and the strobile begins to dry (Figure 2).

Figure 2. Phenological stages of male strobili of *Pseudotsuga menziesii* (Mirb.) Franco.

From the phenological data of each sampled branch, the beginning and end date in Julian days (number of days from January 1st) was determined, as well as the duration of the period of receptivity of the female structures and pollen dispersal.

Formation of study groups

For this investigation, the trees evaluated were formed into two groups. In the Group 1, those trees that formed reproductive structures with all treatments were included, but not necessarily with the presence of both types of strobili within the same tree, because floral induction treatments do not always promote the formation of both sexes. In the second, only data from five trees from the first treatment were integrated, because they had female and male structures in the same tree (Group 2), in addition to completing all stages.

Data analysis

To determine the beginning, end and duration dates of each of the phenological stages, the SYNCHRO macro (Zas *et al.*, 2003) of the SAS® statistical analysis program (SAS, 2013) was used, with which it was possible to obtain the phenological overlap (*PO*) indexes. Also, the dates of the beginning, end and duration of female receptivity and pollen dispersal among all trees were correlated using Spearman correlation coefficients (*r*) (de Winter *et al.*, 2016).

In order to determine the relationship of the phenological events of the flowering of both types of structures with the environmental temperature, the maximum and minimum temperatures recorded for the *Amecameca* municipality on the AccuWeather (2022) website were used during the study period (January-March 2022) to calculate the degree-days (*DD*) accumulated from the first day of January until the end of the receptivity period of the female structures; the following equation was used:

$$DD = \left[\frac{max\ T° + min\ T°}{2}\right] - treshold\ T°$$

Where:

DD = Degree-days

Max T° = Maximum daily temperature (°C)

Min T° = Minimum daily temperature (°C)

Threshold T° = Lower threshold temperature of the species (°C)

Based on the work of Ebell and Schmidt (1964), the threshold temperature of *P. menziesii* was determined to be 10 °C in natural populations of British Columbia, Canada.

Results

Variation in reproductive phenology

The period of receptivity of the female reproductive structures among all the trees of the studied groups was the same, with an average duration of 14 days (Table 1). The average beginning date of receptivity (E3) was recorded on day 57 (February 26th) and ended on day 70 (March 11th). The period of maximum individual receptivity varied from 6 to 21 days (Table 1, E4).

Table 1. Number of days from January 1st in which female receptivity and pollen dispersal occurred in *Pseudotsuga menziesii* (Mirb.) Franco.

		Group 1			Group 2		
		Minimum date	**Maximum date**	**Mean**	**Minimum date**	**Maximum date**	**Mean**
Female receptivity	Beginning	52.5	60.5	56.5	52.5	60.5	56.5
	End	66.5	73.5	70	66.5	73.5	70
	Duration (days)	6	21	14	6	21	14
Pollen dispersal	Beginning	52.5	64.5	58.5	60.5	60.5	60.5
	End	60.5	66.5	63.5	66.5	66.5	66.5
	Duration (days)	6	6	6	6	6	6

The tree pollen dispersal period of the groups lasted six days on average (Table 1). The beginning of the pollen dispersal period (E4) in Group 1 occurred on day 59 (February 28th), while in Group 2 it occurred two days later (March 2nd). The end of this stage (E4) was on March 5th in Group 1, and in Group 2 a couple of days later.

Relationship of flowering phenological events with degree-day accumulation

The onset of average female receptivity (E3 and E4) occurred when an average of 335 *DD* were accumulated (Table 2), while the average pollen dispersal (E4) occurred when 348 *DD* accumulated (Table 2).

Table 2. Average, maximum and minimum values of accumulated degree-days for the mean female receptivity (E3 and E4) and pollen dispersal (E4) of *Pseudotsuga menziesii* in *Amecameca*, State of Mexico.

		Mínimum *DD*	**Maximum *DD***	**Mean *DD***
Female receptivity	Beginning	304.0	361.5	335.0
	End	412.5	468.0	436.5
Pollen dispersal	Beginning	304.0	392.5	348.0
	End	361.5	412.5	384.0

Pollen dispersal in both groups of trees began two days on average after female receptivity and lasted for six days. The fact that female receptivity lasts longer is due to the fact that it was considered receptive since E3, that is because between 30 and 40 % of the strobili can be receptive.

Phenological overlap

The receptivity of the female strobili began on day 52 (February 21st) in 20 % of the trees; by day 61, 80 % of the individuals were at their maximum receptivity (E4) for a period of approximately one week, at the same time that 50 % of the trees were dispersing pollen (Figure 3). It was observed that the periods of maximum female receptivity and maximum pollen dispersal coincided phenologically for a six-day period, approximately.

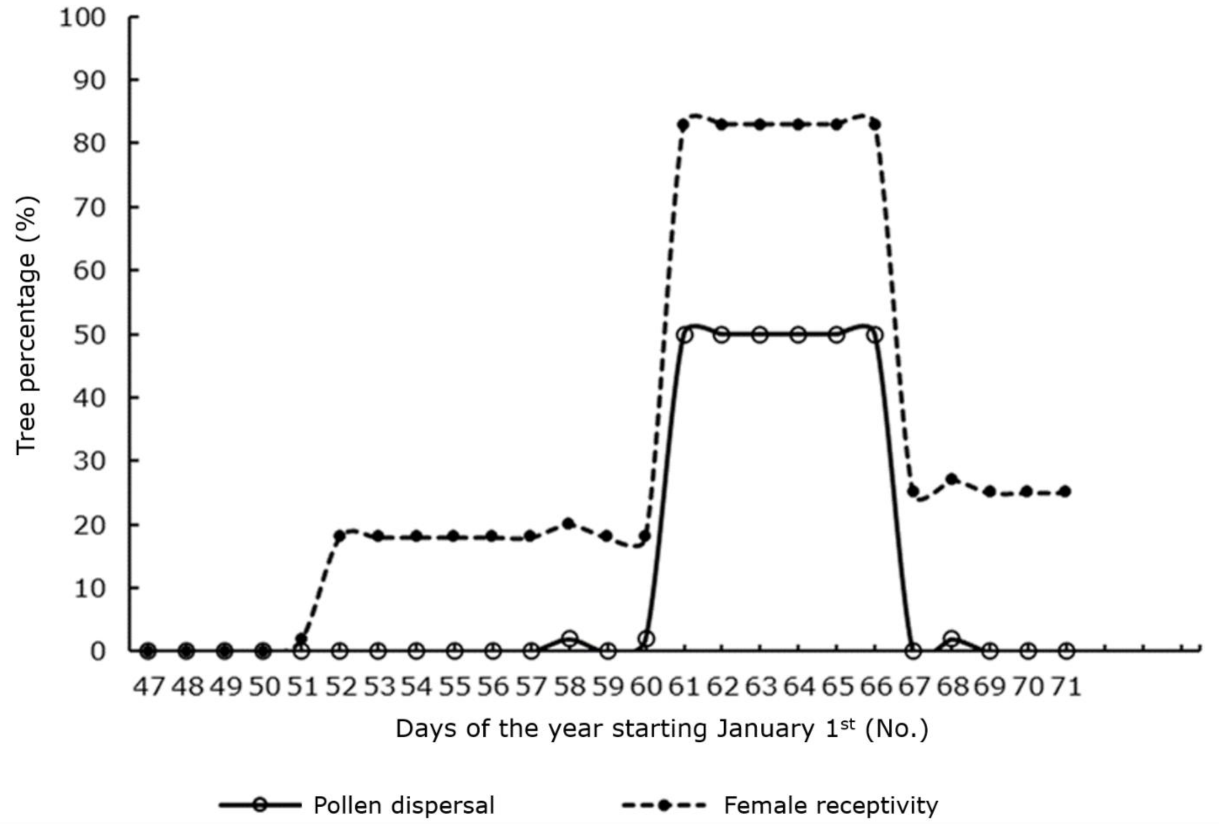

Figure 3. Phenological overlap of female receptivity and pollen dispersal events.

Phenological overlap index (*PO*)

The average *PS* varied from 0.60 to 0.64 in Group 1 and Group 2, respectively, very similar values and moderate to high synchronization. Within Group 1, the average phenological overlap index at the individual level of the two structure types was 0.60, with minimum values of 0.38 and maximum of 0.71 for female structures, while for masculine structures, they were from 0.0 to 1.0. For Group 2, the average phenological overlap index at the individual level for female structures was 0.64 (0.27 to 0.88), and for male structures was 0.64 (0.24 to 0.83) (Table 3).

Table 3. Phenological overlap index (*PO*) between female receptivity and pollen dispersal in *Pseudotsuga menziesii* (Mirb.) Franco trees.

Tree number	**Group 1**		**Group 2**	
	Average female *PO*	**Average male *PO***	**Average female *PO***	***Average male PO***
1	0.38	1.00	-	-
3	0.50	0.67	0.27	0.83
10	0.68	0.61	0.83	0.66
18	0.64	0.39	0.60	0.73
19	0.55	1.00	-	-
27	0.43	1.00	-	-
34	0.60	0.67	0.60	0.73
43	0.58	0.63	-	-
48	0.70	0.00	-	-
55	0.70	0.00	-	-
56	0.67	-	0.88	0.24
69	0.71	0.26	-	-
General	0.60	0.60	0.64	0.64

The correlations between the periods of female receptivity and pollen dispersal were high, positive and significant ($p \leq 0.001$) for the beginning, end and duration dates of the periods (Table 4). However, these results may be overestimated due to the small number of trees used for the analyses, since not all of them formed reproductive structures, or did not complete their development.

Table 4. Spearman correlation coefficient (*p* values) between female receptivity and pollen dispersal of *Pseudotsuga menziesii* (Mirb.) Franco.

		Pollen dispersal		
		Beginning	**End**	**Duration**
Female receptivity	Beginning	0.879 (0.001)	0.879 (0.001)	0.858 (0.003)
	End	0.879 (0.001)	0.879 (0.001)	0.858 (0.003)
	Duration	0.817 (0.007)	0.817 (0.007)	0.796 (0.010)

Discussion

There were no differences between groups of trees, regarding the periods of pollen receptivity and dispersal, but the latter was of shorter duration. Possibly, the stress conditions caused by the girdling of the trunk affected the periods of differentiation of the floral buds, initiation and subsequent development of the male strobili (Webber, 2004).

According to the results, female receptivity begins before pollen dispersal in the same tree, as in *Pinus patula* Schltdl. & Cham. (Hernández *et al.*, 2016), *P. radiata* D. Don. (Griffin, 1984; Kang *et al.*, 2001) and *P. sylvestris* L. (Burczyk and Chalupka, 1997; Sarvas, 1962). This behavior is known as "metandry" (Pulkkinen, 1994), which is common in species of the *Pinus genus*. The average duration of receptivity of the female strobili of *Pseudotsuga menzeisii* in the two groups was 14 days, less than that recorded for the same species by El-Kassaby (1989) in sexually mature and productive trees present in a seed orchard, who documented a duration of 24.3 days in natural conditions and 17.3 days in trees that were under cooling treatment. However, it is shorter than the duration indicated by Owens *et al.* (1981), whose values for female receptivity varied between four and six days.

For pollen dispersal, in the same study (El-Kassaby, 1989) it was documented that under natural conditions and at the population level, this phase lasted 27.7 days to 18.3 days in trees under cooling treatment. The few existing records indicate that the optimal duration of the pollen dispersal period is three to five days (Barner and Christiansen, 1962; El-Kassaby, 1989).

In species such as *Pinus greggii* Engelm. *ex* Parl. (Ruiz-Farfán *et al.*, 2015) and *P. nigra* J. F. Arnold (Alizoti *et al.*, 2010), a phase shift was recorded in the maximum peaks of both phenological events, but the dispersion periods were shorter than those of receptivity. The duration of pollen dispersal can be shortened by excess humidity, due to continuous periods or short events of abundant rain, which causes compaction of the pollen within the strobile and it quickly loses viability. The opposite occurs during droughts when pollen dispersal is faster (Ruiz-Farfán *et al.*, 2015).

The duration of the pollen dispersal period in *Pseudotsuga menziesii* (Erickson and Adams, 1989) and in *Pinus radiata* (Codesido *et al.*, 2005) depends on humidity, temperature and *DD* accumulated during the differentiation process and later development of the reproductive structures (Huusko and Hicks, 2009). For *P.*

menziesii, between 800 and 900 *DD* above 10 °C are required to initiate dispersal at an altitude of 305 m, while at altitudes between 762 and 1 067 m, 400 *DD* are needed (Ebell and Schmidt, 1964). However, in this study 348 *DD* were accumulated. Possibly, the lower number of degree-days required in the plantation that is located at a higher altitude (2 640 m) suggests an adaptive value of the species to initiate the flowering processes.

On the other hand, not all the trees in Group 1 developed both types of reproductive structures in the same tree; all of them presented female strobili, but only in 50 % were male strobili observed. Therefore, the participation of not all individuals in the formation of male strobili and pollen dispersal is a limitation for the production of viable seeds, since only few trees act as pollinators or pollen dispersers. On the contrary, in Group 2 in which the trees developed female and male strobili on the same tree, they had the possibility of crossing each other, with slightly higher values regarding the average synchronization index (*PS*=0.64).

The average *PS* between female receptivity and pollen dispersal in both groups was 0.60 and 0.64, which is interpreted as moderate to high synchronization. This is possibly explained by the little variability in the periods of pollen receptivity and dispersal, however, the pollen from the trees in Group 2 had a greater probability of pollinating all individuals. These values are higher than those recorded in other conifers such as *P. patula* and *P. greggii* in 2012, which were 0.48 and 0.57, respectively (Ruiz-Farfán *et al.*, 2015; Hernández *et al.*, 2016).

The correlations between the periods of female receptivity and pollen dispersal ($0.79 \leq r \leq 0.87$) indicate that the phenological events are not independent, since there are probably effects associated with the treatments to promote early flowering that affect its subsequent development; however, in the same plantation the formation of reproductive structures was observed in untreated trees of the same age. Lower values were recorded in *Pseudotsuga menziesii* (El-Kassaby *et al.*, 1984), *Pinus patula* (Muñoz-Gutiérrez *et al.*, 2019) and *P. taeda* L. (Askew, 1988) with positive correlations between 0.45 to 0.67. The fact that phenological events are not independent favors self-fertilization, which negatively affects seed production (Muñoz-Gutiérrez *et al.*, 2019).

Variation in strobile production and synchronization has been observed in species of the *Pinus* genus and other conifers regularly at early ages, and in first generation seed orchards (El-Kassaby *et al.*, 1988; Matziris, 1994). In these cases, the use of growth regulators and controlled crossings are alternatives to maximize the participation of the individuals of interest.

Conclusions

There are no broad differences in reproductive phenology between groups of trees, particularly, the quantity and maturation of male strobili are not equal. This fact gives the opportunity for a group of trees to pollinate several trees with varied receptivity, since they are moderately synchronized, despite the fact that the female receptivity stage (E3) begins earlier and lasts longer than pollen dispersal (E4).

The *PS* and the correlation values between the phenological events of the development of both reproductive structures in the studied groups are high, if the origin of the plantation material (seed) is considered.

The use of growth regulators and cultural practices to promote early flowering in young trees are useful techniques to reduce seed production times, and do favor the phenological overlap of reproductive structures between trees. However, trees within the same plantation, not included in the present investigation, were detected with a certain precocity to the formation of female and male reproductive structures, similar to the evaluated groups; therefore, complementary management activities, such as artificial pollination, are operationally required.

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Conflict of interests

The authors declare that there are no conflicts of interest. Dr. Liliana Muñoz-Gutiérrez declares that she has not participated in the editorial process of this manuscript.

Contribution by author

Sara Irene Velasco-Hernández: field data collection, statistical analysis and interpretation of results and writing of the manuscript; Liliana Muñoz-Gutiérrez: execution and supervision of the research, statistical analysis and interpretation of results and correction of the manuscript; J. Jesús Vargas-Hernández and Mario Castelán Lorenzo: review, complement and correction of the manuscript.

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