

Article

Cambial Activity and Phenology in Three Understory Species along an Altitude Gradient in Mexico

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Abstract: The aims of this study were to evaluate the cambial activity and phenology of three species with different life forms (*Alchemilla procumbens*, *Acaena elongata* and *Ribes ciliatum*) along an altitudinal gradient and to establish which environmental variables (light, soil humidity and temperature) had the greatest influence on cambial activity and phenological stages. Over two years, data on phenology, growth and cambium were gathered every four weeks in three to six sites per species in Sierra Nevada, Mexico. The results showed that *Ribes* is the only species that terminates cambial activity with leaves senescence and is influenced by the minimum soil temperature. The light environment influenced the vegetative stages in *Alchemilla* (cryptophyte), while in *Acaena* (hemicryptophyte), the mean soil temperature explained the findings related to leaf area during the dry season and growth along the gradient. In the three species, the reproductive phase dominated for a longer period at higher elevations, especially in *Alchemilla*. Only *Ribes*, the phanerophyte, showed a similar cambial activity to other trees and shrubs. Although cambium reactivates during the dry season, no xylogenesis occurs. The three species varied during the time in which vascular cambium was active, and this was dependent on the altitude. Specifically, the variation was more rhythmic in *Ribes* and switched on and off in *Alchemilla*. It is likely that, depending on the life form, vascular cambium may be more or less susceptible to one or more climate factors.

Keywords: vascular cambium; life-forms; leaf area; soil humidity; soil temperature; global light factor; indirect light factor; dry season; cryptophyte



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1. Introduction

It is well known that, in temperate regions, the activation of the vascular cambium of tree species is closely related to the development of leaves due to increases in the environmental temperature [1,2]. Meanwhile, in tropical and subtropical regions, the reactivation of the cambium is mostly associated with water availability [3,4]. Seasonality is also a determining factor in phenological events and cambial activity [5,6].

Phenological stages (bud break, leaf development, flowering, fruiting, and dormancy) are used in different models to predict future climate change at a continental scale [7]. In addition, the phenological stages of plants and cambial activity have been shown to be related to environmental factors such as temperature, soil humidity, precipitation and photoperiod [8]. The phenological changes associated with climatic factors and seasonal periods occur simultaneously with radial growth, a product of cambial activity, suggesting that phenological events may promote the onset of cell division in the vascular cambium (VC) [6,9]. This correlation has been recorded in both temperate [10–12] and tropical [3,4,13] tree species. High temperatures promote the onset of cambial activity [14] and phenophases [15], while periods of higher levels of precipitation and light hours induce increased cell division in the VC [16].

Phenological events and cambial activity can be modified along altitudinal gradients. For example, it has been reported that temperature causes delays in the phenostages of woody species as the altitude increases [17]. With respect to cambial activity, Zhang et al. [18] reported that xylogenesis started 8 days earlier with each 100 m increase in elevation. In contrast, Moser et al. [19] found a 3- to 4-day delay in cambial activity and cell elongation per 100 m increase in elevation. Both phenological stages and cambial activity are dynamic and plastic, and species adjust to the environmental conditions of each locality [20]. Most studies of cambial activity and phenology have focused on canopy trees [4,5,16], with few studies having been conducted for understory trees or shrubs [21–24].

Plants in the understory are important for maintaining structural and ecosystem functions [25]. The understory mediates carbon dynamics and energy flow, influencing the cycling rates of nutrients such as nitrogen, phosphorus, potassium, and Mg [26], while sustaining diverse biotic interactions [27]. The occurrence and diversity of species in the understory may be affected by the heterogeneity of the physical and chemical characteristics of the soil, as well as light [28]. The understory contains the greatest diversity of life-forms [29,30]. However, limited studies have been carried out on this important group of plants. Most studies of the different life-forms of the understory have focused on diversity and reproductive phenology in comparison with invasive species, climate change and anthropogenic disturbances [28,31,32]. However, knowledge on the relationship between phenology stage and cambial activity in association with site environmental factors is lacking, with most of the studies conducted having focused on small trees or shrubs [24,25].

Therefore, it is expected that phenology and cambial activity will change with increases in altitude, due to variations in light, temperature, and humidity. The aims of the present work were to evaluate how cambial activity and phenology are driven by altitude in three species with different life forms. Additionally, we aimed to establish which of the environmental variables—soil moisture, soil temperature and light—has the greatest influence on the phenostage and cambial periodicity.

2. Materials and Methods

2.1. Study Site

The study area is located on the western slope of Tlaloc mountain (19°23′43″ and 1°28′37″ N, 98°42′51″ and 98°48′12″ W, 4100 m asl), in the morphotectonic province known as the Trans-Mexican Volcanic Belt, situated in the northern region of Sierra Nevada, Mexico. This region experiences a humid–temperate climate, with a rainy season from May to October and a dry season with isolated rain from November to April. The mean precipitation level is between 900 and 1000 mm and the mean annual temperature ranges between 6.5 and 10 °C, with a thermal oscillation of 5 to 7 °C [33,34]. The soils are dark gray–brown to very dark, deep, variable in organic matter (7% to 36%), have a medium texture (loam or sandy-loam), and a hydrogen ion potential (pH) ranging from 4.5 to 6.3. The study area comprises six types of vegetation with oak forests being located at the lowest altitudes and alpine grassland at the highest, with a few species distributed throughout the range [35,36].

Six sites were selected along an altitudinal gradient from 3098 to 3963 m asl, and phenology and cambial activity were monitored monthly for each of the three species selected. *Alchemilla procumbens* Rose is a cryptophyte and the most commonly distributed species along the gradient (3098–3963 m asl). This species is an evergreen runner, 0.24 to 0.73 m long with erect branches 9.46 to 26.42 cm tall, where terminal reproductive structures are present. *Acaena elongata* L. is an evergreen hemicryptophyte that grows from 3098 to 3630 m altitude and is a highly ramified shrub that measures 0.44 to 1.15 m tall. *Ribes ciliatum* Humb. & Bonpl. ex Roem. & Schult. is a phanerophyte which commonly grows as a small tree up to 3.20 m tall and is brevi-deciduous, with a distribution that is restricted by the altitudinal gradient (3098 to 3550 m asl). For each of the three selected species (here

after, *Alchemilla*, *Acaena*, and *Ribes*), five visually healthy individuals were marked with color rings at each site and monitored at intervals of 4 weeks for 2 years.

2.2. Environmental Factors

At each site, soil temperature and moisture data were collected with Dataloggers (Extech Instruments) buried 15 cm deep into the soil and in the vicinity of the selected individuals studied. The Dataloggers collected temperature and humidity data at 30 min intervals, 24 h per month, and the minimum, maximum and mean values were calculated for each variable (Supplementary Table S1). The light environment for each selected plant was inferred from hemispherical photographs taken directly at the location of each individual plant at 1 m from the ground above the crown of *Alchemilla* and *Acaena* and at more than 3 m for *Ribes*. The photographs were taken with a Nikon camera fitted with a hemispherical lens with a 180° angle-of-view, oriented in the direction of magnetic north, and mounted on a self-leveling tripod. Hemispheric photographs were taken before sunrise, sunset, and under overcast days. The photographs were analyzed with the HEMIVIEW® version 2.1 software (Delta-T Devices Ltd., Cambridge, UK), configured with latitude, longitude, and altitude data of each site and the date of each photograph. The variables selected for light environment analyses were: the direct site factor (DSF), indirect site factor (ISF), and global site factor (GSF). The DSF is the proportion of direct sunlight relative to that in the open, while the ISF is the proportion of diffuse skylight, and the GSF is the total proportion of global radiation on each plant relative to that in the open. The GSF is a weighted sum of the ISF and DSF which comprises the total radiation each individual plant is exposed to in the understory [37,38]. The mean, maximum and minimum values were calculated per month for each site and each species (Supplementary Tables S2–S4).

2.3. Phenology, Leaf Area and Growth

To evaluate phenology, we adopted the BBCH (Biologische Bundesanstalt, BUNDessortenamt and Chemical industry) identification scale for the main growth stages [39], namely, bud, leaf and shoot development, inflorescence emergence, flowering, fruit development and ripening, and the beginning of dormancy (Table 1). It is important to mention that for *Alchemilla*, the development of the stolons was indirectly followed by the development of aerial branches (Figure S1). The predominant phenological stage per individual was recorded every 4 weeks. For each individual, a percentage of phenostage was assigned depending on the magnitude of the pheno-event, as suggested by Fournier [40]. The scale varied from 0 to 4, where 0 indicates absence of the stage, 1 refers to presence of the stage with a magnitude ranging from 1 to 25%, 2 equates to a magnitude ranging from 26 to 50%, 3 indicates a magnitude ranging from 51 to 75% and 4 infers a magnitude of 76 to 100%.

Leaf area was evaluated for the leaves present during the rainy season (May), which corresponds to leaf development (11) and those produced during the dry season (November), which corresponds to the beginning of dormancy, characterized by new leaves with smaller laminas (92, Table 1). Fifteen leaves were collected from five individuals per site per species for both months and leaf area was measured with an LI-3100c integrator (LI-COR, Lincoln, NE, USA). Growth was monitored through internode elongation per site per species every four weeks for five branches (identified using colored rings) per individual. The relative growth rate (RGR) was calculated using the equation: $RGR = (\ln L_2 - \ln L_1) / (t_2 - t_1)$ by Hunt [41], where L_2 is the length (cm) of the internode in t_2 and L_1 is the length (cm) of the internode in t_1 . The time elapsed between measurements was four weeks; therefore, RGR is given in cm week^{-1} .

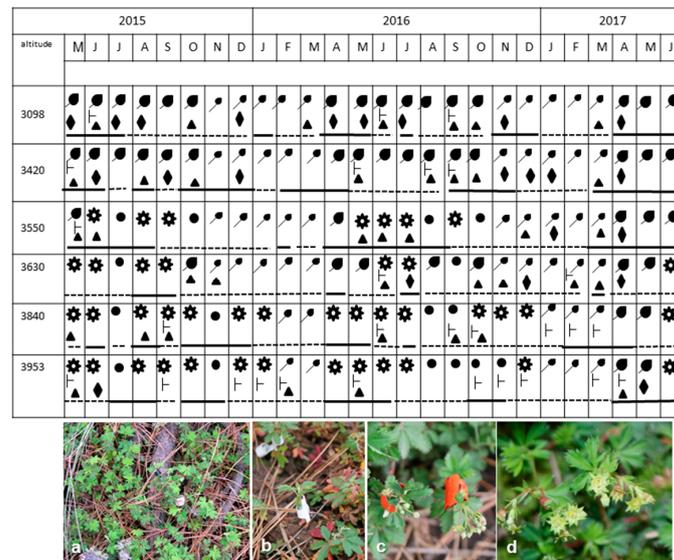


Figure 1. Phenology and cambial activity of *Alchemilla procumbens* over the altitudinal gradient, Sierra Nevada, Mexico (May 2015–June 2017). Phenostage with >50% is represented by month. Bud break (07) ▲, leaf development (11) ◆, leaf development (19) ●, branch growth (31/3) †, flowering (65) ☀, development of fruit (71/79) ●, dormancy (92) ⚭. Cambium: active — inactive - - - - - (a–d) = different phenological phases. (a,b) leaf development and branch growth, (c) flowering, (d) development of fruit.

Table 1. Phenological phases determined for the three species studied, coded according to Meier [30]. Some of these phenophases are illustrated in Figures 1–3.

Phase	Code	Phenophase
▲	07	Beginning of bud break (first green leaf tips just visible)
◆	11	Leaf development (first leaves unfolded)
●	19	Leaf development (first leaves fully expanded)
†	31/3	Beginning of shoot or branch growth (axes)
▼	55	Inflorescence emergence (flower buds visible)
☀	65	Flowering
●	71/79	Development of fruit
●	89	Ripening
⚭	91	Beginning of dormancy, beginning of axillary bud formation
⚭	92	Beginning of dormancy, new leaves with smaller lamina

Cambial activity was evaluated in the branches of two neighboring plants of the monitored individuals with the same phenostage. Two branches of 1 to 3 years of age were cut with a handsaw or fine tooth saw per individual per site monthly. Samples 0.5–1.0 cm long were immediately fixed with Craff II [42]. In the laboratory, the samples were washed with running water and smaller segments (2 × 2 mm) were made using disposable microtome knives and placed in Eppendorf tubes in 50% ethanol. Then, the samples were dehydrated and embedded in methyl acrylate (Technovit7100, Kulzer, Wehrheim, Germany), in accordance with Yañez-Espinosa et al. [9]. Transverse and radial sections 0.5

to 1 μm thick for *Alchemilla* and 2 to 3 μm thick for *Acaena* and *Ribes* were cut in a rotary microtome (RM2125 Leica, Westlar, Germany), stained with cresyl violet—following the method of Zarlavsky [43]—and mounted with synthetic resin (Hycel, Monterrey, Mexico). We considered the vascular cambium to be active when there were divisions towards the phloem or xylem, very thin walls and mitotic figures were detected [15,44,45]. The cambium was inactive when there were no divisions, the fibers in the xylem had a narrower radial diameter and thicker walls, and the sieve tube elements in the phloem were partially collapsed, with a greater callose abundance also being observed.

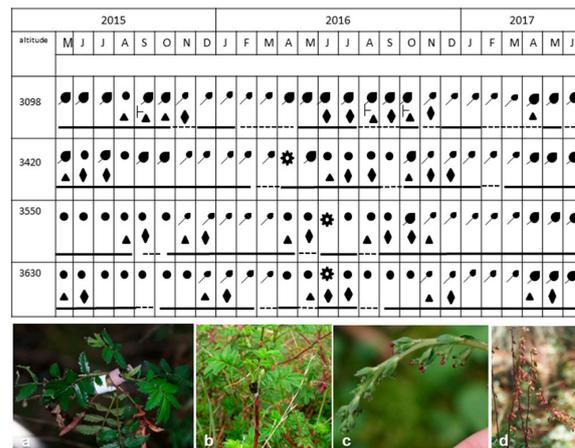


Figure 2. Phenology and cambial activity of *Acaena elongata* over the altitudinal gradient, Sierra Nevada, Mexico (May 2015–June 2017). Phenostage with >50% is represented by month. Bud break (07) ▲, leaf development (11) ◆, leaf development (19) ●, branch growth (31/3) †, flowering (65) ⚙, development of fruit (71/79) ●, dormancy (92) ⚡. Cambium: active ——— inactive - - - - - (a–d) = different phenological phases. (a,b) leaf development and branch growth, (c) flowering, (d) ripening.

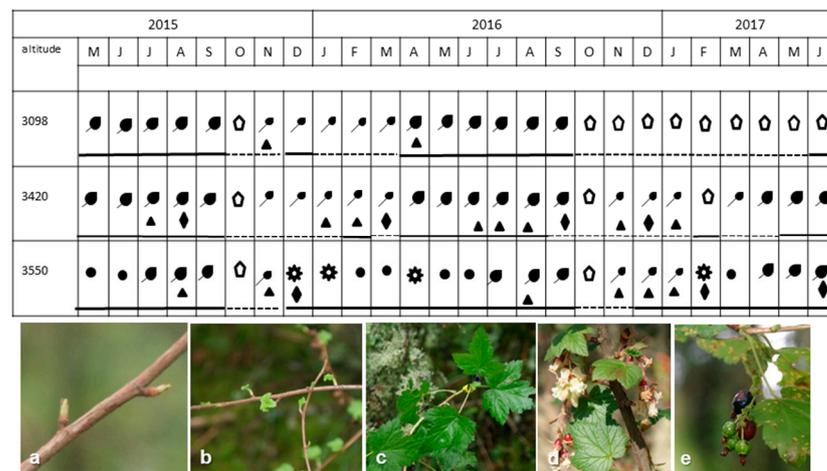


Figure 3. Phenology and cambial activity of *Ribes ciliatum* over the altitudinal gradient, Sierra Nevada, Mexico (May 2015–June 2017). Phenostage with >50% is represented by month. Bud break (07) ▲, leaf development (11) ◆, leaf development (19) ●, branch growth (31/3) †, flowering (65) ⚙, development of fruit (71/79) ●, dormancy (92) ⚡. Cambium: active ——— inactive - - - - - (a–e) = different phenological phases. (a) bud-dormancy, (b) bud beak, (c) leaf development, (d) flowering, (e) fruit development.

2.4. Cambial Activity

2.5. Statistical Analyses

The data of cambial activity, phenology, leaf area and relative rate growth, as well as the environmental variables (light, soil temperature and soil humidity) were transformed to improve linear relationships and normal distributions. Most values were transformed with \log_{10} , while the values expressed in percentage were transformed with arcsine [46]. A constant of 10 was added to all the variables to eliminate the values of zero in both transformations. For each species, a generalized canonical correlation analysis was performed to examine the relationships between two sets of variables and to measure the intensity of the relationships, while also delineating the canonical correlation. For the canonical correlation between the dependent variables (cambial activity, growth, leaf area rainy season, leaf area dry season, budburst, leaf expansion, vegetative, flowering, fruiting) and independent (mean values of temperature, humidity, light), only those that had the highest values in the first five standardized canonical variates are reported here. A *t*-test was also performed to evaluate if differences existed between the leaf areas of leaves growing in November and May. Additionally, a variance analysis was employed to detect differences for both leaves developed in November and May along the gradient. All the analyses were conducted with SAS version 9.1 [47].

3. Results

3.1. Cambial Activity

Along the altitudinal gradient, the three species showed variable time lapses in which the vascular cambium (VC) was active (Figures 1–4). This was particularly marked for *Alchemilla* and *Acaena*. The species that showed the highest periods of cambial inactivity was *Alchemilla* (Figures 1 and 4A). *Acaena* showed longer periods of cambial activity, except at lower altitudes (3098 m, Figures 2 and 4B) and in October–November, the VC in *Ribes* was inactive in the three altitudes where the species is distributed (Figures 3 and 4C).

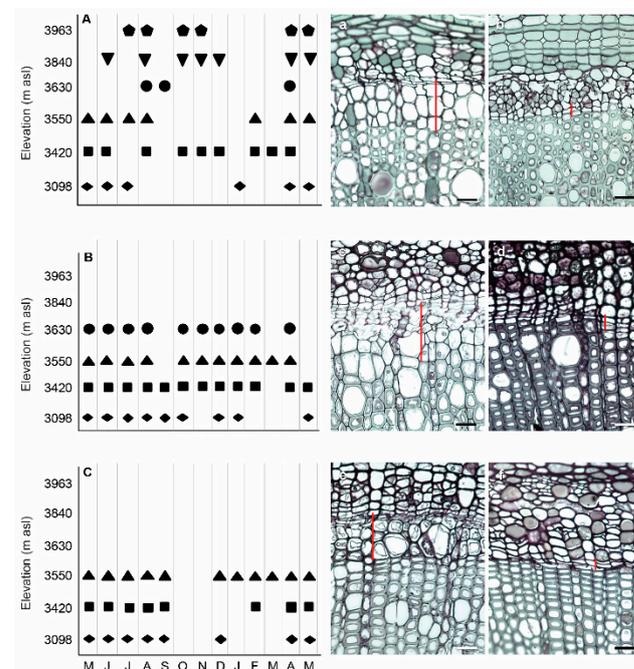


Figure 4. Cambial activity in the three species over the altitudinal gradient summarized for one year, May 2015 to May 2016. (A) *Alchemilla procumbens*, (B) *Acaena elongata*, (C) *Ribes ciliatum*. Symbols represent active cambium at each altitude. (a,c,e) = active cambium plus xylogenesis (vertical red line); (b,d,f) = inactive cambium (vertical red line). Scale bar is 20 μ m.

3.2. Phenology

In *Alchemilla*, leaf development and branching growth consistently predominated in individuals at low altitudes (3098 and 3420 m) all year around, whereas flowering and fruiting dominated at higher altitudes (3840 and 3963 m, Figure 1). *Acaena* had longer periods of leaf development and branching growth, with a single flowering–fruiting period at 3098 m. However, as in *Alchemilla*, flowering–fruiting predominated in the individuals at higher altitudes (3550 and 3630 m, Figure 2). In *Ribes*, the vegetative phase was predominant at the intermediate altitude of its distribution (3420 m), while towards the higher altitude, the vegetative stage showed a reduction (Figure 3). In addition, *Ribes* lost its leaves in October of 2016 and the following month began to expand the leaves of the dry season; however, at lower altitudes (3098 m) in 2017, the buds were maintained until June (Figure 3). Flowering and fruiting were only observed at the highest altitude (3350 m).

The phenological observations allowed us to recognize that *Acaena* and *Alchemilla* renew their leaves throughout the year and never remain without foliage; on the other hand, in *Ribes*, the leaves fall for a short period (nearly a month (Figure 3)). The three species showed a significant decrement in the leaf area in the leaves produced from November to March. These leaves were present in the three species during the dry season and correspond to the dormancy phase. The leaves with a higher leaf area were produced during the rainy season and a Student's *t*-test found significant differences between the leaves produced at the beginning of dormancy—starting in the dry season—for all pairs compared for one year (Table 2). In addition, differences along the gradient exist between the three species (*Alchemilla*: $F = 167.97$, $df 5$, $p < 0.0001$; *Acaena*: $F = 127.98$, $df 3$, $p < 0.0001$; *Ribes*: $F = 6.11$, $df 2$, $p < 0.0001$) for some altitudes (Table 2).

Table 2. Mean and standard deviation of leaf area produced during the rainy season (May, M), which corresponds to first leaves fully expanded, and leaves produced during the dry season (November, N), which developed at the start of dormancy along the altitudinal gradient. Underline mean for each pair within each altitude per species indicates differences found in the Student's *t*-test. Capital letters indicate significance ($p < 0.05$) for leaf development in May; lower case letters indicate significance ($p < 0.05$) for the area of leaves that developed at the start of dormancy.

3098 Mixed Forest		3420 Fir Forest		3550 Fir Forest		3630 Pine Forest		3840 Pine Forest–Alpine Grassland		3963 Alpine Grassland	
N	M	N	M	N	M	N	M	N	M	N	M
<u>0.89</u> ^a ± 0.30	1.17 ^B ± 0.43	<u>0.40</u> ^d ± 0.11	1.45 ^B ± 0.34	<u>0.78</u> ^b ± 0.11	0.80 ^C ± 0.12	<u>0.88</u> ^a ± 0.24	2.35 ^A ± 0.17	<u>0.50</u> ^c ± 0.06	0.75 ^C ± 0.17	<u>0.56</u> ^c ± 0.08	0.63 ^D ± 0.11
<u>1.23</u> ^c ± 0.53	3.33 ^B ± 2.12	<u>1.53</u> ^b ± 0.89	3.21 ^B ± 1.63	<u>1.81</u> ^a ± 0.49	4.44 ^A ± 1.06	<u>0.64</u> ^d ± 0.14	4.48 ^A ± 2.11				
<u>7.83</u> ^b ± 1.34	12.1 ^A ± 3.32	<u>12.48</u> ^a ± 1.25	13.78 ^A ± 2.80	<u>3.45</u> ^c ± 1.75	13.53 ^A ± 3.48						

3.3. Effect of Environmental Variables on Vascular Cambium and Phenology

The results of the canonical correlation analysis by species showed that five canonical functions explain more than 90% of the total variance (Table 3). In *Alchemilla*, the light variables (ISFmin and GSFmean) strongly and inversely influenced the initiation of the growth of new branches, as indicated by the first canonical variate (Table 4). That is, at higher altitudes, *Alchemilla* exhibited a shorter vegetative phase lifespan for flowering and fruiting, whereas at lower altitudes (3098 m), the vegetative phase predominates throughout the year. The second canonical variate indicates that the minimum soil temperature influenced the beginning of dormancy which was associated with a smaller leaf area (Table 4, Figure 1). The following three canonical variables explain 18% of the variance (Table 3), with a positive correlation between cambial activity and the minimum soil moisture level (Table 4). In *Acaena*, the first two canonical variables—mean soil temperature and the light environment (DSFmin and GSFmax)—had a positive effect on the relative growth rate and the beginning of dormancy, with leaves showing a smaller leaf area (Table 5, Figure 2), while cambial activity was influenced by the maximum soil humidity (the fifth canonical variable (Table 5)). For *Ribes*, the first two canonical variables explain 77% of the variance (Table 3). The minimum soil temperature has a positive effect on cambial activity and a

negative effect on the beginning of dormancy, with a smaller leaf area in the first canonical variable (Table 6, Figure 3). In contrast, ripening is positively influenced by the maximum soil temperatures.

Table 3. Eigenvalues/percentage of variance and F Wilk's lambda ($p < 0.0001$) for each species studied.

Canonical Function	Eigenvalues/% Variance		
	<i>Acaena</i> F = 10.81	<i>Alchemilla</i> F = 7.94	<i>Ribes</i> F = 9.88
1	3.16/39	2.60/59	3.92/38
2	1.93/23	0.78/18	3.39/33
3	0.97/12	0.40/9	1.31/13
4	0.82/10	0.22/5	0.77/7
5	0.56/7	0.17/4	0.55/5

Table 4. Canonical cross-loadings of the first five pairs of canonical variables of environment with cambium and phenology of *Alchemilla procumbens*. Bold indicates the environmental or plant variables with the highest values.

Environment	V1	V2	V3	V4	V5
Temperature mean	0.669	−0.326	0.182	0.068	0.032
Temperature minimum	0.517	−0.274	0.190	0.096	0.093
Humidity maximum	0.190	0.141	0.052	−0.375	0.164
Direct Site Factor minimum	−0.046	0.572	0.073	−0.056	−0.058
Direct Site Factor maximum	0.014	0.198	−0.417	−0.025	−0.043
Global Site Factor minimum	0.073	0.565	0.011	−0.117	−0.189
Plants	W1	W2	W3	W4	W5
Cambium	−0.081	0.149	0.213	−0.312	0.412
Beginning of bud break (07)	0.118	−0.089	0.085	0.477	0.156
New leaves with smaller lamina (92)	−0.700	−0.033	0.130	−0.144	−0.058
Relative growth rate	0.704	0.150	0.365	0.024	0.037
Beginning of new branches (31)	−0.339	−0.581	−0.030	−0.009	0.094
Inflorescence emergence (55)	−0.004	−0.160	0.470	0.215	0.132

Table 5. Canonical cross-loadings of the first five pairs of canonical variables of environment with cambium and phenology of *Acaena elongata*. Bold indicates the environmental or plant variables with the highest values.

Environment	V1	V2	V3	V4	V5
Temperature minimum	0.257	0.524	−0.139	0.033	0.051
Temperature maximum	0.155	−0.18	0.100	−0.281	0.001
Humidity minimum	−0.011	0.176	0.057	0.317	0.018
Humidity maximum	−0.053	0.059	0.080	0.113	0.174
Indirect Site Factor minimum	0.568	−0.351	0.143	−0.065	0.076
Global Site Factor mean	0.562	−0.394	−0.086	−0.048	−0.016
Plants	W1	W2	W3	W4	W5
Cambium	−0.082	0.355	0.201	0.257	−0.079
Leaf development (fully expanded, 19)	0.077	0.194	0.312	−0.046	0.179
New leaves with smaller lamina (92)	−0.552	−0.435	0.009	−0.013	−0.069
Beginning of new branches (31)	−0.571	0.193	−0.139	0.038	0.056
Developing of fruits (71/79)	0.449	0.021	−0.271	−0.001	−0.250

Table 6. Canonical cross-loadings of the first five pairs of canonical variables of environment with cambium and phenology of *Ribes ciliatum*. Bold indicates the environmental or plant variables with the highest values.

Environment	V1	V2	V3	V4	V5
Temperature mean	0.481	0.093	−0.476	0.059	0.170
Temperature minimum	0.588	−0.124	−0.193	0.069	0.208
Temperature maximum	0.231	0.500	−0.416	0.021	0.099
Humidity mean	0.474	−0.216	0.449	−0.001	−0.007
Direct Site Factor mean	0.022	0.436	−0.035	0.107	− 0.323
Direct Site Factor maximum	0.047	0.319	−0.011	0.119	− 0.324
Indirect Site Factor mean	−0.225	0.430	0.138	0.250	0.225
Plants	W1	W2	W3	W4	W5
Cambium	0.748	0.348	−0.020	−0.068	−0.181
Beginning of bud break (07)	0.392	−0.282	− 0.497	−0.103	−0.085
New leaves with smaller lamina (92)	− 0.700	−0.065	0.201	−0.166	−0.279
Relative growth rate	0.219	0.295	−0.370	0.329	−0.015
Inflorescence emergence (55)	−0.216	−0.152	0.493	0.065	−0.104
Ripening (89)	−0.319	0.631	−0.274	0.202	0.098

4. Discussion

This work describes—for the first time—the cambial activity and phenology of three species with different life forms (cryptophyte, hemicryptophyte, and phanerophyte) as well as how soil temperature, soil humidity, and light influenced them. The vascular cambium in *Alchemilla* (cryptophyte) and *Acaena* (hemicryptophyte) species did not show the same pattern of seasonality as other temperate region species. However, both *Alchemilla* and *Acaena* showed a longer reproductive stage period at higher altitudes, which was associated with reactivation of the VC without xylogenesis. Meanwhile, *Ribes* exhibited a VC pattern that was similar to other tree and shrub species of temperate forests. Notably, the minimum soil temperatures stimulate budburst and development of new leaves in *Ribes* during the dry season, after a short period of deciduousness when dormancy starts. The minimum temperatures did not fall as drastically as in other regions with temperate forests and maintained optimal conditions for the development of new phloem cells or the completion of differentiation of phloem mother cells.

4.1. Seasonality

Phenological stages have frequently been related to the seasonal cycles of the environment and biological responses. For example, in spring–summer, flowering, fruiting, vigorous growth, leaf development and maturation [48,49] take place. Meanwhile, in autumn–winter, leaf senescence occurs and there is no growth. However, this seasonality (reproductive) was only noticeable in the *Alchemilla* species coming from the intermediate sites (3550 and 3630 m) and in the individuals of *Acaena* present at 3420 m asl. *Ribes* presented seasonality in the period of leaf senescence along its distribution. This contrasts with what has been reported for species from alpine and subalpine areas that have a marked seasonality that allows them to avoid climatic stressors [50].

4.2. Phenology

In the three species studied, there was a reduction in the duration of the vegetative phase as the duration of the reproductive phase increased, especially in *Alchemilla* and *Acaena*. The negative correlation between the vegetative and reproductive stages has been described in perennial species and is attributed to the high cost of resources required to produce fruits and seeds, resulting in plants stopping growing and developing new leaves [51]. The longest periods in the reproductive stage were found at higher altitudes. In shrub species, constant fruit production has already been recorded and is related to the moisture content of the soil [32]. The high fruit production level has been related to the nutrients available in the soil and their dilution with the increased humidity levels [52]. In

In addition to this, the high synchrony of *Acaena* with fruiting is a pattern that is related to the opportunistic strategy of this species to anthropogenic disturbances [32,52]. In our study site, this species is present in open areas and where light gaps in the fir and pine forests occur along the gradient. In *Alchemilla*, flowering was observed as the predominant stage, while the fruiting stage was dominant in *Acaena*. The intense production of flowers in alpine species has been associated with the high accumulation of non-structural carbohydrates [53] and this may be the case for both cryptophyte and hemicryptophyte species at higher altitudes. In *Acaena*, flowering occurs relatively quickly, while fruiting is a very long process because the ripening of the fruit requires more time. In contrast, *Ribes* showed a fruiting period of two months, preceded by two months of flowering. The reproduction phase was only observed at the higher limit of *Ribes*' distribution along the gradient, suggesting that this species finds the fir forest conditions optimal (light, soil humidity and temperature) for reproduction.

4.3. Vascular Cambium

The onset and termination of cambial activity was different in the three species studied along the altitudinal gradient. In *Ribes* (phanerophyte), VC behaves in a similar manner to other shrub and arboreal species, namely it has a synchronous termination of cambial activity with leaf abscission [2]. However, the onset of activity was related to the budburst of new leaves, starting in the dry season in October–November; however, no xylogenesis occurred [54].

For *Alchemilla*, a seasonally defined pattern of switching VC on and off was not observed. Several authors have mentioned that the high temperatures of early spring promote the reactivation of VC after being turned off in winter [15,55]. However, Rossi et al. [55] pointed out that air temperatures of 5.6 to 8.5 °C promote the formation of new xylem cells. Alternatively, Liang et al. [16] established that minimum temperatures must be found above 0 °C. The maximum soil temperatures at the lower altitude sites (<3350 m) throughout the year are above 10 °C, and the air temperatures are between 5.2 and 9.8 °C (unpublished data), which would allow the activation of the VC independent of the season of the year. It should be noted that Rossi et al. [55] make reference to the fact that soil temperatures do not affect the formation of new cells in the VC of trees. However, *Alchemilla*—an evergreen stoloniferous species—may be affected by soil temperatures. The time during which VC is active in the individuals of *Alchemilla* at lower altitudes (<3350 m) is greater than that at higher altitudes (>3630 m). The variation in soil temperatures and humidity is higher at higher altitudes in the gradient studied and may explain why the cambium may turn on and off throughout the year in *Alchemilla*. *Acaena* showed the longest periods of cambial activity compared to *Alchemilla* and *Ribes*. This phenomenon has already been reported in tropical tree species [5] and in species growing in deciduous dry forests [3].

In the three species, it was observed that the periods in which VC was active during the first year varied with respect to the reactivation and duration of the second year. This change in cambial activity has been related to changes in temperature between years [56].

4.4. Vascular Cambium, Phenology and Environmental Variables

As Yañez-Espinosa et al. [13] concluded, multivariate analyses identify the relationships among the phenology and cambial activity of species with climate, microclimate and soil factors. Here, the canonical correlation analyses revealed that different variables of soil temperature and humidity, as well as light, differentially influence the phenology and cambium of the three understory species studied. Cambial activity and the presence of leaves during the dry season (those occurring during dormancy) were strongly correlated to the minimum soil temperature in *Ribes*, which at the highest site was below 3 °C, with a humidity greater than 90% when fruiting is present. It is likely that either the phloem mother cells complete their differentiation process, or new cells are developed and the translocation of photo-assimilates favors the occurrence of such leaves and the reproductive phase. The relationship between temperature and cambial activity displayed by *Ribes* has

previously been reported in other trees species [14]. On the other hand, in *Acaena*, the leaves of the dry season showed a high correlation with the average soil temperature. The relationship between leaves of the dry season and soil temperature has not been reported previously. Bonilla-Valencia et al. [32] mentioned that the various phenophases in *Acaena* are affected by the soil organic matter content, soil moisture, light and canopy opening in fir forests. We also found that DSF has an effect on branching and the relative growth of this species.

It has been recorded that lower temperatures induce delays in leaf expansion [2], while maximum temperatures have been mostly related to spring leaves [57], with this being in agreement with the three species studied. The leaves that developed in May during the rainy season had a larger area along the gradient and this is also when xylogenesis occurred [54]. Notably, in *Alchemilla*, a cryptophyte, the main drivers for growth are global and indirect light levels (ISF_{min} and GSF_{mean}). This is due to the fact that the vascular cambium switches on and off in order to maintain phloem conduction almost all year round, allowing the species to have smaller leaves and reproductive structures during the dry season. This also corresponds with the lower temperatures. This ability to reactivate the cambium, in particular, the stimulation of divisions towards the phloem or complete phloem mother cell differentiation when the temperature and humidity environmental conditions are optimal, probably favors the success of this species along the gradient. As mentioned above, the reproductive phase predominates at higher altitudes but almost no reproduction was observed at lower altitudes (3098 m). Future studies to understand the consequences of losing reproduction at lower latitudes must be conducted.

At the site level, the variables in vertical gradients are modified by the structure and density of the canopy, which is why the microclimate is considered to have a greater influence on cambial activity, radial growth and tissue differentiation [13]. The differences in VC and xylem production are related to the variations in the microclimate associated with the types of vegetation along the gradient, as previously reported by Jiménez-Noriega et al. [54], who mentioned the variation in the onset and termination of xylogenesis in individuals from various altitudes. In this work, the three species studied—with different life forms—presented associations of different magnitudes and meaning between cambial activation, phenological events and environmental variables across the altitudinal gradient studied.

5. Conclusions

The variation between the phenological phases and vascular cambium activity of the individuals of the three species suggests that they are closely related to the environmental variables on each site along the altitudinal gradient. This study presents a novel finding in the fact that, along this gradient, two (cryptophyte, hemicryptophyte) of the three species maintain their leaves all year around, and only *Ribes* is brevi-deciduous. The three species have small leaves during the dry season, which also corresponds to the lower temperatures along the gradient. Future studies should focus on the phloem conductivity that is associated with cambial activity, interpreted here as the differentiation of mother cells or divisions towards the phloem. On the other hand, three trends in phenology along the altitudinal gradient were clear, especially in the two species with the greatest distribution: *Alchemilla* (cryptophyte) and *Acaena* (hemicryptophyte). Namely, these trends refer to the predominance of the vegetative phase at lower altitudes, with alternations between the reproductive and vegetative stages in relation to seasonality at the intermediate sites, in addition to the predominance of the reproductive stage at higher altitudes. Prolonged reproductive phenology has the potential to generate reduced growth rates, even at the expense of survival, since a significant proportion of energy is devoted to sexual reproduction. However, it should be considered that sexual reproduction promotes genetic variability and induces greater chances of survival in the face of adverse or stressful environmental factors, especially for species that occur at high altitudes. The trends, variations and similarities that the species presented in terms of phenology and cambial activity had

not previously been reported. This study represents a precedent for understanding the differential responses of the different life-forms found along altitudinal gradients, where clear changes in the environment are present.

Supplementary Materials: The following are available at <https://www.mdpi.com/article/10.3390/f12040506/s1>, Table S1: Values for soil temperature (T) and humidity (H) from March 2016 to April 2017 over the altitudinal gradient in Sierra Nevada, Mexico, Table S2: Values for direct site factor (DSF) from May 2016 to April 2017 over the altitudinal gradient in Sierra Nevada, Mexico, Table S3: Values for indirect site factor (ISF) from May 2016 to April 2017 over the altitudinal gradient in Sierra Nevada, Mexico, Table S4: Values for global site factor (GSF) from May 2016 to April 2017 over the altitudinal gradient in Sierra Nevada, Mexico, Figure S1: *Alchemilla procumbens*. (A) Plant with ring to monitored monthly. (B) Complete plant showing dead and new aerial branches with leaves. (C) Detail of new branches (arrows).

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Abbreviations

BBCH Biologische Bundesanstalt, Bundessortenamt and Chemical industry, DSF direct site factor, GSF global site factor, ISF indirect site factor, Mg magnesium, RGR relative growth rate, VC vascular cambium.

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