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Vegetative rescue and *in vitro* propagation of *Persea willdenovii*

Rescate vegetativo y propagación *in vitro* de *Persea willdenovii*

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SUMMARY

Persea willdenovii is a native species popularly known for its high potential for medicinal use. Due to problems in low seed production and germination, vegetative propagation appears promising. Thus, we seek with this work to evaluate techniques of vegetative rescue and the potential use of shoots obtained in tissue culture. Adult trees of the species were used to apply total and partial girdling treatments and obtain pruned branches as vegetative rescue techniques. Shoot production was monitored over time (90 to 240 days). Sprouts were used for *in vitro* propagation, and disinfestation treatments were carried out using biocides such as NaClO and Plant Preservative Mixture™ (PPM™), changing the time and concentrations of the products. Multiplication was also tested using doses of 6-benzylaminopurine (BAP) and media and the potential for callogenesis under combinations of BAP and Naphthalene Acetic Acid (ANA). The vegetative rescue by complete or semi-girdling has the potential to produce shoots, though pruned branches proved to be one of the best techniques for rescue. In the micropropagation, the use of biocides such NaOCl for a long period of time (2 % for 20 min) with PPM™ added to the culture medium shows a potential for disinfestation in the *in vitro* establishment. For *in vitro* multiplication, the usage of WPM and BAP (2 a 4 g L^{-1}) promotes higher shoot length, shoot number and leaf number. For indirect organogenesis by leaf segments, the cultivation with BAP and ANA was not responsive to the induction of callogenesis.

Keywords: vegetative propagation, girdling, pruned branches, micropropagation.

RESUMEN

Persea willdenovii es una especie nativa popularmente conocida por su uso medicinal. Debido a problemas en la baja producción y germinación de semillas, la propagación vegetativa parece promisoria. Consecuentemente, en este trabajo se evaluaron técnicas de rescate vegetativo y el uso potencial de brotes obtenidos en cultivo de tejidos. Se usaron árboles adultos de la especie para realizar tratamientos de anillo total y parcial, y obtener ramas podadas como técnicas de rescate vegetativo. La producción de brotes se controló a lo largo del tiempo (90 a 240 días). Se utilizaron brotes para la propagación *in vitro*, y los tratamientos de desinfestación se llevaron a cabo utilizando biocidas como NaClO y Mezcla Conservante de Plantas (PPM™), cambiando el tiempo y las concentraciones de los productos. La multiplicación también se probó utilizando dosis de BAP y medios y el potencial de callogénesis en combinaciones de 6-Bencilaminopurina (BAP) y ácido naftalenacético (ANA). El rescate vegetativo mediante anillo completo o parcial tiene el potencial de producir brotes, pero las ramas podadas demostraron ser una de las mejores técnicas. En la micropropagación, el uso de biocidas como el NaOCl durante un período prolongado (2 % durante 20 min) con PPM™ añadido al medio de cultivo muestra un potencial de desinfestación en el establecimiento *in vitro*. Para la multiplicación *in vitro*, el uso de WPM y BAP (2 a 4 g L^{-1}) promueve una mayor longitud y número de brotes, y número de hojas. Para la organogénesis indirecta por segmentos de hojas, el cultivo con BAP y ANA no respondieron a la inducción de callogénesis.

Palabras clave: propagación vegetativa, anillo, ramas podadas, micropropagación.

INTRODUCTION

Persea willdenovii Kosterm, popularly known as bush avocado, maçaranduba, pink cinnamon or pau de Andrade belongs to the Lauraceae family. The species is known for its diversity of uses in urban forestry, civil building, and medical properties (Batista *et al*. 2010). The local population uses the bark of this species to make tea as treatment of gastric ulcers and wound healing. These claims were supported by pharmaceutical and phytochemical studies due to the presence of mucilaginous structures that are related to protective action on inflamed mucous (Rosa *et al*. 2017).

Despite its high potential, *Persea willdenovii* is present in several endangered species lists and is considered critically endangered in the Red List of Rio Grande do Sul state's flora (CNCFlora 2020), mainly due to the exploratory use in popular medicine through removal of the bark

with the consequential death of individuals in the forests. In addition to this problem, the species has an irregular fruit maturation which can compromise the quality of seeds and storage potential, making the species more vulnerable to human actions and therefore more susceptible to the extinction process. The vegetative propagation of species that present low seed germination and / or recalcitrant seeds, such as *Persea willdenovii,* can ensure the preservation of the species in addition to multiplying individuals with desirable traits for commercial purposes (Fior *et al.* 2007).

Studies conducted on aspects of propagation of *Persea willdenovii* by Fior *et al*. (2007) presented limitations, mainly related to restriction in the morphogenic potential, presence of endogenous contamination and high tissue oxidation. There are many factors influencing the success of the propagation such as genotype, maturation, type of explant, age and size of the explant, and extrinsic factors such as plant regulators and culture medium (Lee and Pijut 2017, Silva *et al*. 2020).

Few species are easily propagated using mature (old material) propagules. In the case of tree species, this difficulty is directly linked to the process of maturation, because the older the plant, the more reduced the cell division rates and regenerative capacity (Nascimento *et al*. 2018), necessary to rejuvenate the mother plant before starting any propagation work. In addition, there is an increase of inhibitors (anatomical barriers, lignification, gibberellins, phenols) and consequently decrease of rooting as the age of the plant increases (Bisognin *et al*. 2015). In woody forest species the gradient of youth grows towards the base of the tree. The basal region of the tree is younger in the ontogenetic age and this is due to the later formation of the apical meristems (terminal regions) in relation to the basal meristems in a tree. It is important to know this ontogenetic behavior of the different parts of the tree, since, depending on the location of the removal of vegetative material (part of the plant), it will certainly interfere in the rooting potential of the propagule and consequently in the production of seedlings (Stuepp *et al*. 2018).

The *in vitro* culture may be an important strategy in solving problems not only in propagation but also in genetic improving and plant biotechnology, especially in perennial woody species (Warakagoda and Subasinghe 2013). The *in vitro* culture of *Persea willdenovii* is still not well explored and has potential among other techniques of asexual propagation, however, there are hindrances related to low morphogenic potential and explant contamination (Fior *et al*. 2007). According to Oliveira *et al*. (2013), high contamination of native tree species is common, since they have superior lignification, endogenous microorganisms, often adverse climatic conditions, and difficulty in carrying out preventive treatment.

Various parts of the mother plant can be used as source for the explant. The choice of material can be influenced by factors such as: availability of the material, contamination level, juvenility of the tissue, among others. The nutritional requirements can also vary, as well as the type and efficiency of the phytoregulators (Cid and Teixeira 2010). However, it is important to highlight that the success of the micropropagation, regardless of the explant used, is subject to the effect of the genotype from the mother plant in response to the stimulus *in vitro* (Stein *et al.* 2009).

To identify and characterize relevant aspects in the production of plants, we conducted a series of experiments with vegetative rescue and *in vitro* propagation. We aimed at verifying if the species has the possibility of vegetative rescue by girdling or pruned branches and at assessing the potential for establishment and cultivation *in vitro*. We are looking for a new propagation alternative for the species that has problems with seed germination. Our hypothesis is that it is possible to use rescue techniques to produce juvenile shoots that will be used in *in vitro* propagation, and that in this environment the species has the possibility of establishment and multiplication, thus allowing to produce clonal plants with sanitary quality, and still seek new studies exploring the medicinal characteristics of the species.

Thus, the objectives of the study are to determine the best method for vegetative rescue in adult plants and evaluate various protocols of *in vitro* propagation of the rescue material of *Persea willdenovii*, a species with great potential popular interest, mainly in medicinal functions.

METHODS

Vegetative rescue. To conduct the study, adult plants (reproductive age) of *Persea willdenovii* located in a native area in the municipality of Urupema, Santa Catarina state, Brazil, were studied. The study area is located in the Catarinense mountain range at approximately 1,400 m a.s.l., at the coordinates of 28° 17’ 38’’ S and 49° 55’ 54’’ W.

This region has a humid temperate climate (Cfb), with average annual precipitation around 1,800 mm, well distributed throughout the year. The average annual temperature is 13 °C, with a very distinct winter and summer. The average temperature for these two seasons is 8 °C and 18 °C, respectively. As characteristics of the region, there may be snow and minimum temperatures of up to -14 °C (EMBRAPA, 1998).The region belongs to the altomontana mixed ombrophilous forest, characterized by the presence of the species *Araucaria angustifolia* (Bertol) Kutze (from 1,000 m a.s.l.), forming groupis in association with other species.

The vegetative rescue was conducted in August 2015. The treatments were: girdling (100 %), semi-girdling at 75 % of the trunk circumference, semi-girdling at 50 % of the trunk and the induction of shoots from pruned branches. The trunk girdling and semi-girdling consisted in the removal of an approximately 2 cm wide bark ring (figure 1A) at a height of approximately 30 cm from the soil, made by sectioning two transverse rows with a machete, to break the bark without damaging the wood. A total of se-

ven trees were used in each treatment (girdling and semi-girdling). The experiment was conducted in a completely randomized system.

For the epicormic sprouts induction treatment from pruned branches, branches were removed, from five mother trees, in the lowest position of the canopy to minimize the effects of maturation and ontogenetic age. Ten branches per mother trees were cut to an approximate size of 1-meter-long and had their ends protected with plastic bags to avoid water loss (figure 1B). The material was placed in a mini-tunnel under a greenhouse (33 % of light blocking), with constant relative humidity (80 %) and an average temperature of 25 °C, in the forest nursery of the State University of Santa Catarina (city of Lages, Santa Catalina state, Brazil).

The evaluations and harvesting of the sprouts were held from the first signal of shooting onwards, at 90, 120, 150, 180, 210 and 240 days (December 2015 to May 2016) after application of vegetative rescue treatments. After each harvest, the number and size (length) of these sprouts were registered, and afterwards the percentage of sprouting per harvest was calculated. For the pruned branches technique, evaluations were made in relation to the length of the branches (sprout number m^{-1}) as done by Nascimento *et al*. (2018).

In the field, diameter at breast height (cm), height (m) and the amount of incident light (lux) in the trunk base of each tree were measured (table 1). An amount of light was measured with a portable digital light meter (Minipa MLM-1011), with three measures interspersed between trees. The assessment was always carried out with a clear sky, without the sun being covered by any cloud. This reading (incident light) was repeated in all harvests (90, 120, 150, 180, 210 and 240 days).

In vitro propagation. For the *in vitro* propagation of *Persea willdenovii*, the sprouts originated from the vegetative rescue (pruned branches or girdling). Figure 2 shows a summary of the micropropagation process carried out, summarizing the experiments in each phase. During the production and harvest of sprouts, the calcium oxychloride based fungicide, bactericide and sporicide Frexus® was

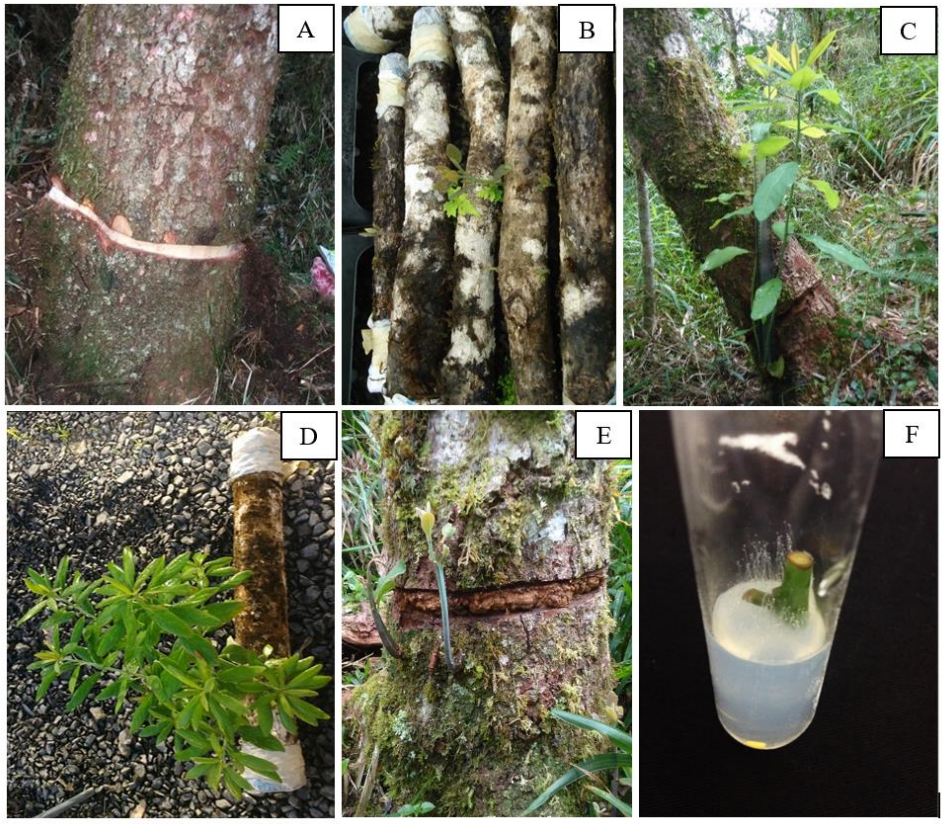

Figure 1. Vegetative rescue and *in vitro* propagation of *Persea willdenovii*. A) girdling in an adult tree in the field, B) pruned branches starting to sprout, C) sprouts in field trees submitted to girdling, D) sprouts on pruned branches, E) healing in the girdling region and F) explant *in vitro* showing contamination.

Rescate vegetativo y propagación *in vitro* de *Persea willdenovii*. A) anillo en un árbol adulto en el campo; B) ramas podadas que comienzan a brotar; C) brotes en árboles de campo sometidos a anillo; D) brotes en ramas podadas; E) curación en la región de anillo; y F) explante *in vitro* que muestra contaminación.

Table 1. List of *Persea willdenovii* trees submitted to vegetative rescue treatments with their respective values: percentage of girdling applied to the circumference of the trunk - treatment (%), tree height (m), diameter at breast height (DBH) (cm) and incident light (lux).

Árboles de *Persea willdenovii* sometidos a tratamientos de rescate vegetativo con sus respectivos valores: porcentaje de anillos aplicadas a la circunferencia del tronco - tratamiento (%); altura del árbol (m); diámetro a la altura del pecho (cm) y luz incidente (lux).

Mother plant	Treatment - % of girdling	Height (m)	DBH (cm)	Average of incident light (lux)
1	100	7.9	24.8	230 (40)*
2	50	12.3	40.7	190 (30)
3	75	13.1	32.8	166 (30)
4	100	6.7	16.5	300 (50)
5	50	6.6	20.5	250 (40)
6	100	4.5	5.7	8,500 (1,200)
7	50	4.7	18.0	250 (30)
8	75	7.8	18.5	300 (30)
9	100	6.4	20.0	360 (50)
10	100	9.3	28.5	2,800 (300)
11	75	9.5	23.7	1,900 (240)
12	50	7.5	24.5	100 (20)
13	75	12.0	29.0	300 (40)
14	50	11.5	42.0	810 (90)
15	75	13.2	31.4	900 (110)
16	100	6.8	20.5	7,030 (900)
17	50	7.1	39.0	200 (30)
18	75	8.4	28.5	245 (40)
19	50	11.6	40.0	1,700 (200)
20	75	8.8	26.6	160 (30)
21	100	10.4	18.2	542 (60)
Average	-	8.6	25.8	1,379.5

* Value in parentheses refers to the standard deviation of the evaluations performed at 90, 120, 150, 180, 210 and 240 days.

applied in the vegetative material, at 0.2 g L^{-1} concentration. The transportation of sprouts originated from pruned branches or girdling was made inside a Styrofoam box, with ice at the bottom and covered by paper sheets moisturized with water. The transport to the material processing site did not last more than 10 minutes (pruned branches) and 40 minutes (girdling). The *in vitro* propagation experiments took place in the vegetal micropropagation laboratory at the Agroveterinary Science Center of the State University of Santa Catarina during 2016.

First, the sprouts were superficially washed using neutral detergent and immersed in running water for 15 minutes. Later the material was segmented in smaller sizes (explants) with ± 3 cm (only nodal segments) and taken to a laminar flow cabinet where the explants were disinfected. The culture medium used was the MS (Murashige and Koog 1962) with 50 % of the concentration of the original; 30 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol, 6 g L^{-1} of agar and 1.5 g L^{-1} of activated charcoal were also added to the medium. The recipients were tubes (150 x 25 mm) containing 15 mL of the medium. The pH of the medium was adjusted to 5.8 ± 0.1 before autoclaving at 120 ºC and 1 atm for 20 minutes.

For the disinfestation, three methods for explant were tested: experiment I – concentrations of the disinfectant sodium hypochlorite (NaClO) in six concentrations: 1, 2, 2.5, 3, 4 and 5 % of active chlorine in contact with the explants for 15 minutes. Experiment II – use of PPM™ biocide in two concentrations: a) 1 mL L^{-1} added to the medium and b) agitating the explants for four hours in contact with 5 mL L^{-1} of the product. In the treatment with the addition of the biocide in the medium, the asepsis was carried out with NaClO 2 % (v v^{-1}) for 15 minutes. And in the treatment under agitation, there was no contact with

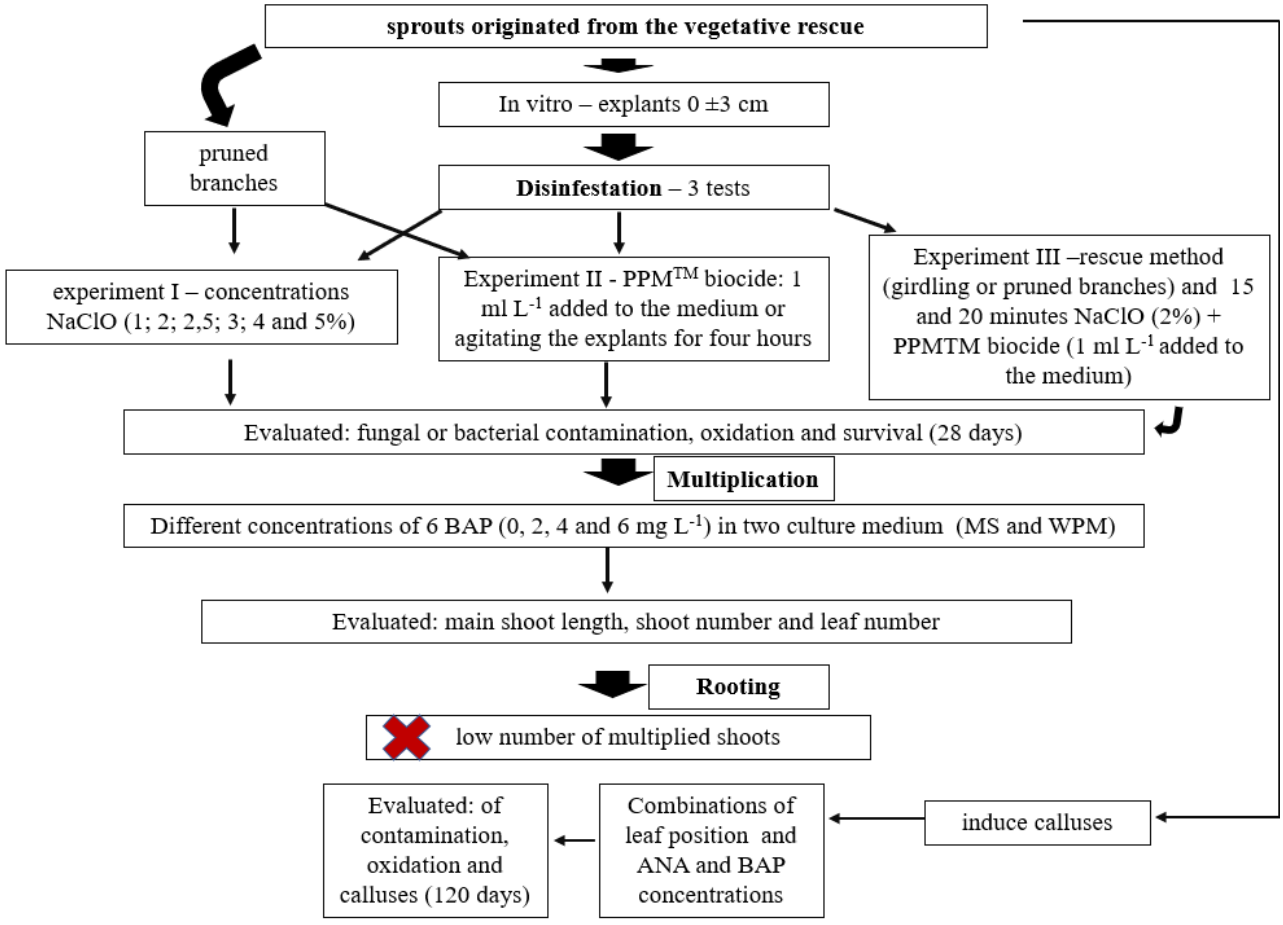

Figure 2. Scheme of the micropropagation process used in *Persea willdenovii*, showing steps from the origin of the material to the cultivation.

Esquema del proceso de micropropagación utilizado en *Persea willdenovii*, mostrando las etapas desde el origen del material hasta el cultivo.

another disinfectant; the explants were directly introduced to the medium. For experiments I and II, the vegetative material was obtained exclusively from pruned branches. Experiment III – the material was divided according to the rescue method: coming from the forest (girdling) or the mini-tunnel (pruned branches). These rescue materials were tested at different times of immersion in sodium hypochlorite (2 % active chlorine): 15 and 20 minutes of contact with the explant (%) + PPM™ biocide (1 mL L^{-1} added to the medium). All experiments used six repetitions, containing three explants in each treatment.

The low number of repetitions and non-utilization of a control treatment was established due to the limited number of nodal segments. Despite the technique that originated the material issued a considerable number of sprouts, the space between them was relatively large, resulting in a small sample of explants. After the asepsis, in all experiments, except the agitation in PPM™ biocide, the explants were washed three times in autoclaved distilled water and incubated in the culture medium. The material was kept in the dark for seven days, and after that period cultivated in the culture room under temperature of 25 ± 3 ºC, in a 16-hour photoperiod and 20 µmol m^{-2} s^{-1} photon flux density, provided by cold white fluorescent lamps. The evaluations of all experiments were carried out in a 28-day period, in which, the incidence of fungal or bacterial contamination, oxidation and survival rate of explants were evaluated.

Shoot segments with previously established shoots *in vitro*, 3 ± 0.5 cm in size, were used for multiplication. Different concentrations of 6-benzylaminopurine (BAP) (0, 2, 4 and 6 mg L^{-1}) were analyzed in two culture media, MS and Woody Plant Media WPM (Lloyd and Mccown 1980). All media were supplemented by 30 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 6 g L^{-1} of agar (Sigma Aldrich®). The experimental design was completely randomized in factorial scheme 4 x 2 (4 BAP concentrations and 2 culture medium), six repetitions containing six explants in each treatment. Later, the material was incubated in the culture room as previously described. After 60 days, the main shoot length (mm), shoot number and leaf number were evaluated.

Due to the low number of multiplied shoots, it was not possible to carry out tests involving rooting. However, it was decided to conduct an experiment to induce calluses. The leaves harvested from branches derived from the vegetative rescue were washed in running water and neutral detergent and later segmented in smaller portions (1 cm²). In a laminar flow chamber, the leaf segments were immersed in ethanol 70 % (v v^{-1}) for 1 minute and in sodium hypochlorite solution (NaClO) at 2 % active chloride for 15 minutes, finally rinsed three times in autoclaved distilled water.

The segments were later sectioned in explants of ±1 cm² containing the central leaf nerve and inoculated in abaxial and adaxial leaf positions in 100 mL flasks containing 30 mL MS medium. The mix was supplemented by 30 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 6 g L^{-1} of agar and the treatments were various combinations of BAP and ANA in concentrations of 0 to 12 mg L^{-1} (table 2). The pH of the mix was adjusted to 5.8 ± 0.1 before autoclaving at 120 °C and 1 atm for 20 minutes. The explants were kept in the dark for 10 days, and after this period, cultivated in the culture room. Evaluations of contamination, oxidation and calluses growth were carried out weekly until 120 days after the inoculation. The experimental design was completely randomized, in factorial scheme 2 x 10 (A x D), the factor A composed by leaf position and factor D comprised by the combinations of ANA and BAP concentrations, composed by five repetitions, with four leaf segments in each treatment.

To test the hypothesis that rescue by girdling or pruned branches are good strategies to produce rejuvenating sprouts that can be grown *in vitro*, we first checked the variables to normal distribution (Shapiro's test, $P < 0.05$) and variance homogeneity (Barttlett's test, $P < 0.05$). When necessary, the data were transformed with a boxcox test. After rechecking the data for their fit to the basic requirements, we proceeded with ANOVA (F test, $P < 0.05$), followed by Tukey's mean comparisons test ($P < 0.05$) or regression analysis. For the regression analysis, linear and second order (quadratic) models were tested. Tree height data, diameter at breast height and incident light were correlated by Pearson with the variables of vegetative rescue.

Table 2. Leaf face position and 6-benzylaminopurine (BAP) and Naphthalene Acetic Acid (ANA) concentrations tested in MS culture medium in the induction of *Persea willdenovii* callogenesis.

Posición de la cara de la hoja y concentraciones de 6-Bencilaminopurina (BAP) y ácido naftalenacético (ANA) evaluadas en el medio de cultivo MS en la inducción de la callogénesis de *Persea willdenovii.*

Treatment	Leaf face position	Phytoregulator (mg L^{-1})	
		BAP	ANA
1	Abaxial	0	0
2	Abaxial	4	4
3	Abaxial	8	8
4	Abaxial	12	12
5	Abaxial	0	4
6	Abaxial	0	8
7	Abaxial	0	12
8	Abaxial	4	0
9	Abaxial	8	0
10	Abaxial	12	0
11	Adaxial	0	0
12	Adaxial	4	4
13	Adaxial	8	8
14	Adaxial	12	12
15	Adaxial	0	4
16	Adaxial	0	8
17	Adaxial	0	12
18	Adaxial	4	0
19	Adaxial	8	0
20	Adaxial	12	0

RESULTS

Vegetative rescue. Despite the wide variation in dendrometric values (height and DAP) and light intensity in the trunk base, there was no significant Pearson correlation of data from the mother trees with variables evaluated (percent, number and height of sprouts). For all variables, there was an interaction between the vegetative rescue methods and harvests (days after application of the technique).

The percentage of sprouted trees (figure 3A) presented the major average in the pruned branches. This method was the only one to show sprouts (69 %) 90 days after the application of the treatment. There was sprouting in 100 % of branches in the harvests at 120, 150, 180 and 210 days; only at 240 days there was a decrease of sprouts (84 %).

The highest number of sprouts (12.2) was observed in the pruned branches technique, at 150 days (January) (figure 3B). This harvest date was also superior for girdling 50 % of the trunk (4.1). For the techniques of 100 % girdling and 75 % girdling the larger number of sprouts was observed in February after 180 days (3.5 and 4.6 respectively sprouts). After 180 days there is a reduction in the number of sprouts in all vegetative rescue techniques, and at 240 days there are practically no more shoots. It should be noted that it was decided to use the pruned branch technique in comparison to girdling to verify the production potential of the technique; however, the number of sprouts for pruned branches refers to the number per linear meter (sprout number m^{-1}), while the girdling ones refer to the amount per tree. Therefore, an average test was not used and only a regression analysis, showing a production behavior over time.

For the sprout length, in all rescue vegetative techniques, the largest size was obtained between 150 and 180 days after application of treatments (figure 3C). The treatments of complete girdling and pruned branches (figure 1D) presented the largest sizes of the sprouted branches with averages between 15 - 20 cm in height. Some individuals of the complete girdling generated sprouts of almost 50 cm (figure 1C). In the last evaluation, in addition to the absence or small number of sprouts in many trees, the size was very small. In this last evaluation, it was also possible to observe an intense healing in most trees in the girdling treatments (figure 1E).

In vitro propagation. For the *in vitro* establishment, experiments I (concentrations of the disinfectant sodium hypochlorite) and II (PPM™ biocide in two concentrations) did not have significant difference according to the Tukey test ($P < 0.05$). High contamination rate was observed during the four weeks following the removal of material from the dark room to the illuminated environment, between 80 to 92 % of explants had fungal or bacterial contamination (table 3). The oxidation varied between 18 and 33 % and the maximum survival was 20 %.

In experiment III, testing the origin of the rescue material and disinfestation treatments with different times of

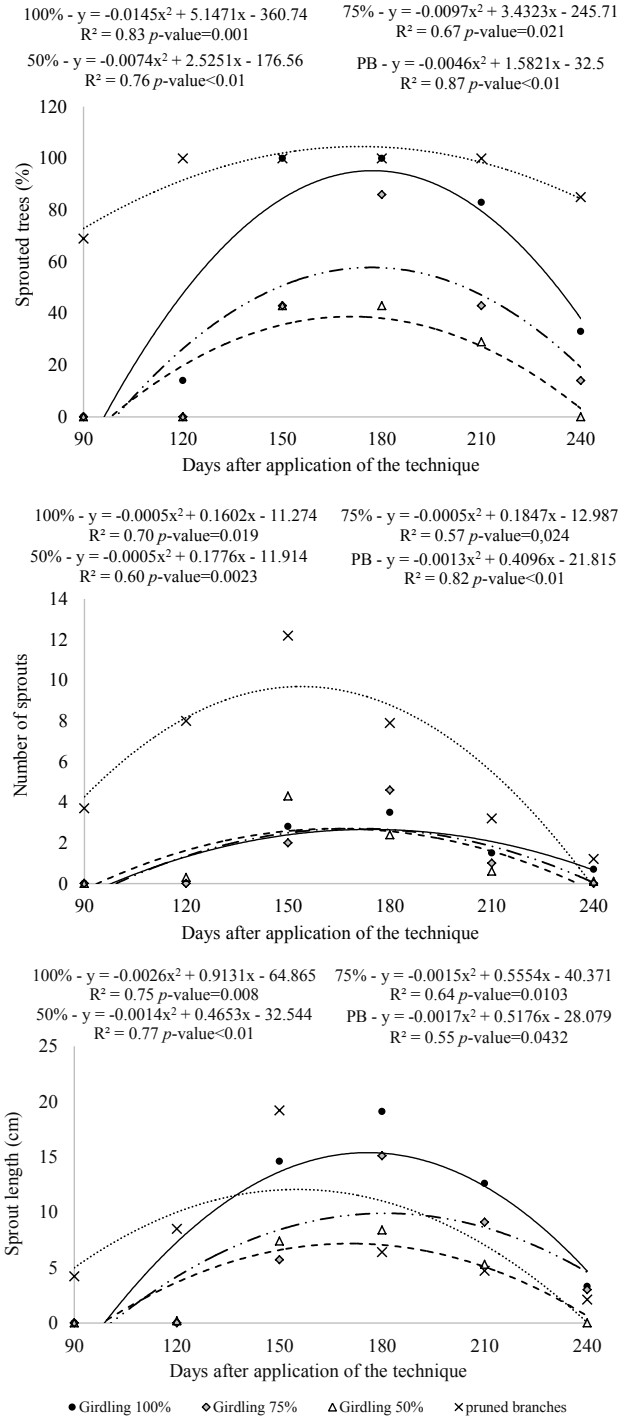

Figure 3. Percentage of sprouted trees (A), average number of sprouts (B) and length of sprouts - cm (C) in *Persea willdenovii* as a function of different vegetative rescue treatments. The days after applying the technique, at which time the assessments took place, correspond to from 90 days (December 2015) up to 240 days (May 2016).

Porcentaje de árboles brotados (A); número promedio de brotes (B) y longitud de los brotes - cm (C) en *Persea willdenovii* en función de diferentes tratamientos de rescate vegetativo. Los días posteriores a la aplicación de la técnica, momento en el que se realizaron las evaluaciones, corresponden a 90 días (diciembre 2015) hasta 240 días (mayo 2016).

NaClO supplemented with PPM™ biocide (1 mL L^{-1}), we observed a difference between treatments. The girdling material presented higher fungal/bacterial contamination (between 82 and 86 %), higher oxidation rate (between 38 and 41 %) and lower explant survival rates (39 to 45 %), regardless of the time of exposure to the NaClO (figure 4). However, the pruned branches material also showed inferior results, similar to those of girdling. The treatment with

Table 3. Fungal / bacterial contamination (%), oxidation (%) and survival (%) in *Persea willdenovii* explants grown *in vitro* after carrying out disinfestation experiments.

Contaminación por hongos / bacterias (%), oxidación (%) y supervivencia (%) en explantes de *Persea willdenovii* cultivados *in vitro* después de realizar experimentos de desinfestación.

		Disinfestation/variables		
Experiment	Treatment	Fungal/ Bacterial contamination (%)	Oxidation (%)	Survival (%)
experiment I – concentrations NaClO	1 %	92^{ns} (8)	33^{ns} (21)	10^{ns} (20)
	2 %	88 (4)	24 (14)	15 (8)
	2.5 %	85 (10)	31 (17)	15 (12)
	3 %	83 (9)	26 (19)	19 (10)
	4 %	89 (10)	30 (10)	7 (8)
	CV (%) (*p*-value)	35.3 (0.843)	19.9 (0.441)	14.4 (0.093)
Experiment II - PPM™ biocide	1 mL L^{-1} added to the medium	80^{ns} (13)	18^{ns} (11)	20^{ns} (10)
	agitating with explants	82 (15)	22 (12)	18 (12)
	CV (%) (*p*-value)	26.4 (0.923)	15.3 (0.192)	16.1 (0.324)

^{ns} - not significant. Values in parentheses represent the standard deviation of the mean.

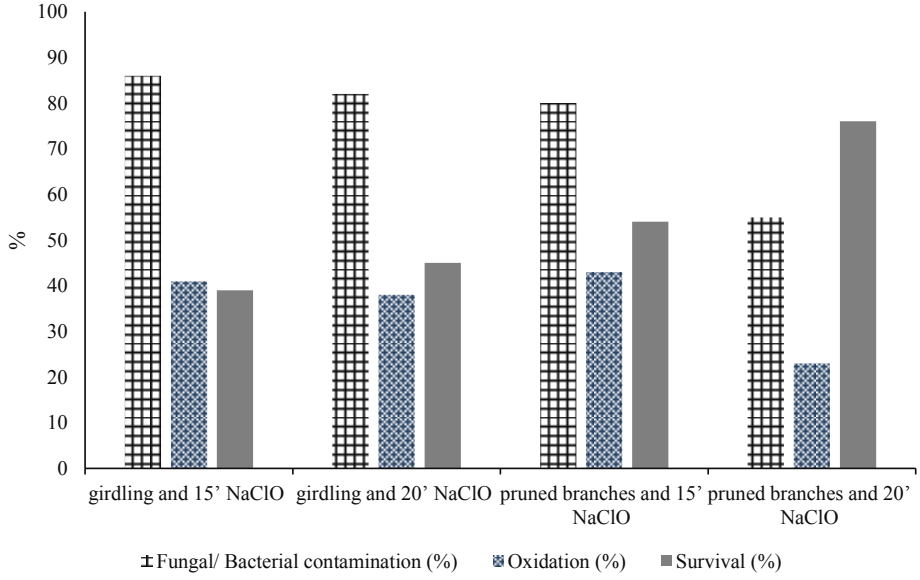

Figure 4. Percentage of fungal / bacterial contamination, oxidation and survival of *Persea willdenovii* explants depending on the rescue method (girdling or pruned branches) and 15 and 20 minutes NaClO (2 %) + PPM™ biocide (1 mL L^{-1} added to the medium) in the *in vitro* establishment. Fungal / bacterial contamination (CV % = 21.3 and *p*-value = 0.021), oxidation (CV % = 18.4 and *p*-value = 0.042) and survival (CV % = 14.2 and *p*-value = 0.0328).

Porcentaje de contaminación fúngica / bacteriana, oxidación y supervivencia de explantes de *Persea willdenovii* en función del método de rescate (anillado o ramas podadas) y 15 y 20 minutos NaClO (2 %) + biocida PPM™ (1 mL L^{-1} agregado al medio) en el establecimiento *in vitro*. Contaminación por hongos / bacterias (CV % = 21,3 y valor-*p* = 0,021), oxidación (CV % = 18,4 y valor-*p* = 0,042) y supervivencia (CV % = 14,2 y valor-*p* = 0,0328).

the best response was pruned branches with exposure for 20 minutes in NaClO. This treatment obtained less contamination (55 %), oxidation (23 %) and higher survival (76 %).

For the *in vitro* multiplication, there was no interaction between factors ($P < 0.05$) (culture medium and BAP concentrations) in the evaluated variables, nonetheless there was a difference between the isolated factors (table 4). For main shoot length (mm) there was a difference between the culture media, in which the WPM presented the highest average (7.5 mm). The addition of BAP showed no difference for this variable. For shoot number, the WPM medium also showed better response (0.51) compared to the MS medium (0.30). The addition of 4 mg L^{-1} represented a larger number of shoots, not differentiating from 2 mg L^{-1}. The absence or the highest concentration (6 mg L^{-1}) of BAP provided low number of shoots. The same behavior (higher averages) was obtained for number of leaves using the WPM medium (0.78) and the concentrations 2 and 4 mg L^{-1} of BAP (1.02 and 0.99).

There was no formation of calluses in the leaf calluses exposed to the evaluated treatments. In general, it was observed that leaves had light green coloration up to 15 days after inoculation, becoming beige after 60 days of cultivation, and after this period, the color changed to brown. After 120 days of *in vitro* cultivation, the explant tissue died, probably due to the lack of favorable conditions for development. The data are not presented because in the evaluation of 120 days, there was no formation of callogenesis or live leaf tissues, regardless of treatment.

Table 4. Main shoot length (mm), shoot number and leaf number depending on concentrations of 6-benzylaminopurine (BAP) and culture medium in *Persea willdenovii* explants multiplied *in vitro*.

Longitud de los brotes principales (mm), número de brotes y número de hojas en función de las concentraciones de 6-bencilaminopurina (BAP) y del medio de cultivo en explantes de *Persea willdenovii* multiplicados *in vitro*.

BAP (mg L^{-1})	MS	WPM	Average
	main shoot length (mm)		
0	5.3 (3.4)	7.1 (2.4)	6.2^{ns}
2	5.1 (2.9)	7.9 (2.0)	6.5
4	6.4 (3.8)	8.3 (4.2)	7.1
6	6.0 (2.5)	6.9 (3.5)	6.4
Average	5.7 B*	7.5 A	
p-value interaction = 0.0963	*p*-value BAP = 0.094 *p*-value medium = 0.023		
	shoot number		
0	0.20 (0.22)	0.40 (0.26)	0.30 b
2	0.35 (0.13)	0.55 (0.27)	0.45 ab
4	0.52 (0.26)	0.73 (0.33)	0.62 a
6	0.14 (0.22)	0.36 (0.15)	0.25 b
Average	0.30 B	0.51 A	
p-value interaction = 0.122	*p*-value BAP = 0.006 *p*-value medium = 0.0162		
	leaf number		
0	0.24 (0.12)	0.41 (0.20)	0.32 a
2	0.72 (0.23)	1.33 (0.37)	1.02 a
4	0.84 (0.31)	1.15 (0.34)	0.99 a
6	0.26 (0.12)	0.24 (0.21)	0.25 b
Average	0.44 B	0.78 A	
p-value interaction = 0.083	*p*-value BAP = 0.001 *p*-value medium = 0.0093		

* Means with different capital letters on the line (culture medium) and lower-case letters on the column (BAP) show a significant difference by the Tukey test at 5 % error. Values in parentheses represent the standard deviation of the mean. ^{ns} - not significant.

DISCUSSION

Vegetative rescue. There was no record of the death of trees subjected to girdling and semi-girdling treatments, meaning that the species supports the removal of a partial or complete girdling of the bark without damaging tree survival. The pruned branches also survived until the end of the evaluations (April 2016), showing a high environmental control of moisture and temperature inside the mini-tunnel, and possession of endogenous reserves, allowing the use of this technique to produce sprouts.

There was no correlation among number of shoots with diameter, height of trees and light intensity. In a work with *Toona ciliata* M. Roem., Pereira *et al.* (2015) did not find correlation between diameter of the mother trees and number of emitted sprouts through vegetative techniques, either. In the same study, dead trees were not observed after vegetative rescue treatments. In contrast, the work of Pinto *et al.* (2013) with *Pterogyne nitens* Tul. presented a positive correlation with the increase in diameter of the plant and increased number and strength of sprouts. There are few published works available to try to understand the influence of the degree of maturation in the growth in diameter and plant height and, consequently, in the production of sprouts. Studies point to some factors prevailing on the regrowth capacity and survival of strains in woody species, including age, level of allocated reserve substances, the genetics of the species and the effect of climate variations throughout the year (Wendling *et al*. 2013, Pereira *et al*. 2017, Nascimento *et al*. 2018).

In relation to climate variations, January and February are the months with a history of higher temperatures in the region (Santa Catarina) and without water deficit problems, which may have contributed to the development of new sprouts. Higher averages of shoots in *Ilex paraguariensis* A. St.-Hil. were also observed in the summer months by Nascimento *et al*. (2018), and this work was carried out in the same experimental area. The fifth and sixth harvests in the field, held in March and April, lowered the production of sprouts, probably the metabolic and physiological cutback of plants due to the decrease in average temperature and temperature range in these months. Another factor that may be responsible for the lower number of sprouts in these harvests was the healing of the girdling part as a response to the application of vegetative rescue (figure 2F). This healing behavior has also been reported to *Toona ciliata* in which the thickness of the bark checked in the ring line may have interfered with the emergency rate of sprouts in some trees (Pereira *et al.* 2015). According to Dias *et al*. (2012), competition for water, nutrients, light and space between sprouts over time may also be related to this reduction.

In addition to the environmental conditions the highest number of sprouts during January and February harvests may be explained by the possibility that these shoots are from gems that had remained dormant after the application of the treatments and did not generate any sprout after the first harvest or, are the product of a morphogenesis, when a cambial cell transforms over time, mainly by the action of auxins (Zahadat *et al.* 2017). According to the same authors, one effect of auxin is to make the bundles of unspecialized stem cells, called cambium cells, which are located near the vascular tissues, transform into vessels. This is especially interesting considering the fact that limited common resources (*e.g.* water) need to be distributed among different branches of a plant via their vessels.

Physiologically, the emission of basal sprouts benefits from the partial or total rupture of apical dominance increasing the cytokinin / auxin ratio (Hartmann *et al.* 2011). Another factor that may have induced the emergence of new sprouts, is stress by girdling, because it may have produced functional disturbances in the trees, which sprouted as a survival strategy. In plants, stress happens by interrupting the transport of photosynthates and other organic metabolites from the higher to the lowest parts in the plant, which is executed by elements and riddled cells, located in the phloem (Taiz and Zeiger 2013). The cut off techniques and stem girdling also managed to induce the emission of basal sprouts in many species such as *Tectona grandis* Linn F. (Badilla *et al.* 2016), *Ilex paraguariensis* (Stuepp *et al.* 2015, Stuepp *et al.* 2016, Nascimento *et al*. 2018), *Sequoia sempervirens* (Pereira *et al*. 2017) and *Toona ciliata* (Pereira *et al.* 2015).

The sprouting ability can also be influenced by factors such as light, thickness and depth of cut (Dinh *et al.* 2018). In this work, we can see that the percentage of girdling at the tree trunk also influences the formation of sprouts, being practically directly proportional in the mother trees of *Persea willdenovii*. For *Toona ciliata* the number of sprouts by annealing is four times higher than those observed in semi-girdling (Pereira *et al.* 2015).

Similarly, vegetative rescue by pruned branches proved to be an effective technique in induction of epicormic sprouts in *Ilex paraguariensis* trees kept in the mini-tunnel (Nascimento *et al.* 2018) and *Eucalyptus cloeziana* F. Muell. trees, where all the pruned branches that were in the greenhouse issued sprouts after 40 days of conditioning (Almeida *et al.* 2007). According to the authors, the induction of epicormic sprouts from pruned branches should be used carefully. Since the way the tissue samples it uses are positioned in the tree, it may mean they are physiologically mature, compromising the juvenility of the material. It is, therefore, important to collect branches of the lower portions of the plant, as these tend to give juvenile shoots. The physiological principle of the method, as well as in stem girdling, is based on shifting the balance between growth regulators (auxin / cytokinin) in support of the issuance of sprouts. Additionally, in most of the angiosperms trees, the dormant epicormic gems, which can be present since the formation of branches / trunk or be the product of morphogenesis, when a cambial cell transforms to yield the new sprouts, are present in the outer bark (Hartmann *et al.* 2011).

The environmental conditions in the mini-tunnel may have contributed to the success of the pruned branches technique. The conditioning in the greenhouse, or similar, creates a favorable microclimate for their survival. In this work, the mini-tunnel has a misting system that keeps the relative humidity at about 80 % and the temperature between 18 and 30 ºC, which may have favored the early sprouting (90 days). For the present study, the meteorological variables of temperature and humidity were not constantly checked, whereas in the study by Nascimento *et al*. (2018) conducted in the same structure, it obtained relative air humidity above 90 % and average temperature between 15 ºC (winter) and 26 ºC (summer). The data was verified with a datalogger. The plastic cover used in greenhouses significantly changes the balance of radiation, when related to the external environment due to the attenuation of incident solar radiation, resulting in a reduction of the internal radiation balance and thus affecting temperature, humidity and evapotranspiration. Vegetative growth is very intense and the growth rate of the aerial part of the plant gradually increases in accordance with increasing temperature.

With the pass of time, after 240 days, shoot emission was reduced, which may be associated with exhaustion of reserves present in the branches. The same was observed in detached branches to induct shoots in *Araucaria angustifolia* (Bertol.) Kuntze and in the experiments of Wendling *et al.* (2009) and *Ilex paraguariensis* (Nascimento *et al.* 2018). The vegetative rescue technique via pruned branches, in addition to getting the best results compared those applied in the field (girdling), has the advantages of having the harvest of material at the plant production site (nursery) and the possibility of higher phytosanitary control of these sprouts.

In vitro propagation. In our study, we found high contamination by fungi / bacteria and oxidation in the first weeks after removing the material that was in the dark at the beginning of the cultivation. Woody plant tissues are quite susceptible to *in vitro* browning, a direct result of stress and chemical reactions induced by polyphenol oxidases and other enzymes. The resulting phenol compounds react with oxygen to form quinine compounds, generally inhibitory of plant growth (Ahmad *et al.* 2013).

Between 80 and 90 % of explants showed contamination in experiment I (NaOCl concentration) and in the second study (PPM™ biocide in two concentrations). The bacterial contaminants manifested after the first week of evaluation, in the base of the explants cultivated *in vitro*, and continued to appear afterwards. In general, the vegetative material survived this bacterial contamination. However, these contaminations generated considerable limitations in the development of the explants. Fior *et al*. (2007) also reported high proportion of contamination by microorganisms of fungal and bacterial origin (74 %) and micropropagation of non-juvenile tissue of *P. willdenovii* during *in vitro* sowing. According to these authors, after various subcultures, the endogenous contamination remained in parts of the material.

In experiment III (establishment) testing the material according to the rescue method and different times of immersion in NaOCl (2 %) + PPM™ biocide (1 mL L^{-1} added to the medium), more promising results were obtained for *in vitro* establishment, mainly of plant material from pruned branches, which were kept in mini-tunnel and presented phytosanitary control with fungicide application. Control with fungicides in mother trees when possible is a good strategy to reduce contamination *in vitro* (Hiti-Bandaralage *et al.* 2017). Better contaminant control in this experiment (III) may also have been due to the addition of PPM™ in the culture medium. Similar results regarding the benefit of using PPM™ were observed in *Calophyllum brasiliense* Cambess., using nodal segments as starting material, where the biocide added to the culture medium reduced the contamination of explants (Silveira *et al.* 2016). According to the same authors, very high concentrations of biocides such as PPM™ and NaOCl can lead to oxidation and mortality.

The difficulties in the *in vitro* establishment of *P. willdenovii* due to contamination and oxidation of the explants continue despite the rejuvenation of the vegetative material; this is probably related to the genetics of the species. Other species of the *Persea genus* also found such obstacles in micropropagation. Studies about the avocado tree (*Persea americana* Mill.) published since 1987 with various cultivars report similar results to the ones found in this (Osorio *et al.* 2018).

As for *in vitro* multiplication, the WPM medium shows better results, with high results of main shoot length (mm), shoot number and leaf number. This may be explained by the difference in salt concentration in the medium. The WPM has fewer salts concentrations (especially nitrogen and potassium) when compared to the MS media, which has high salt concentration, the nitrate and ammonium ions specifically (Rathwell *et al.* 2016).

The use of 2 to 4 mg L^{-1} of BAP promoted higher multiplication, with an increase in the number of sprouts and mainly of new leaves. The absence of the regulator promoted a low rate of regeneration. The same result was obtained in *Melanoxylon brauna* Schott. where the use of between 2 and 4 mg L^{-1} of BAP provided a higher number of shoots (Silva *et al.* 2020). According to Brum *et al*. (2002), the multiplication of sprouts with the use of BAP may be related to the influence of the genetic charge from the mother plant, of the growth regulator used to help with cellular division and in the break of the dormant state of axillary shoots, until then inhibited by apical dormancy. The results obtained in the *in vitro* multiplication of *Persea willdenovii* are promising and show viability of this technique in multiplication and conservation programs of this species. However, newer work must be done aiming for the increase in number of sprouts, exploring, for example, experiments about the influence of the environment

(temperature, light, culture mix solidity, etc.) in the induction of new sprouts.

In the conditions of this work, BAP and ANA phytoregulators and their combinations, in doses of 0 to 12 mL L^{-1}, did not contribute to the callogenesis induction of *Persea willdenovii*, other types of phytoregulators and concentrations pending future testing. Some studies, such as Encina *et al.* (2014) in *Persea americana,* show success of the callogenesis; however, the avocado is a cultivated species, and that generally presents better results *in vitro*. Callus culture is an important technique to increase the number of secondary metabolites in medicinal species (Bansal *et al.* 2013), consequently it should receive further studies with *Persea willdenovii* considering the numerous medicinal properties that the species has.

The rooting test was not possible due to the low number of explants multiplied, owing to the loss of regenerative capacity. Thus, it is recommended to carry out further studies, primarily aimed at improving the multiplication capacity. However, in this experiment it was possible to notice good results regarding the use of cured material (mainly pruned branches), suggesting that these new works follow this methodology.

CONCLUSIONS

The vegetative rescue by complete or semi-girdling has the potential to produce shoots to be used in the vegetative propagation of *Persea willdenovii*. The technique of pruned branches proved to be one of the best techniques for rescue, because in addition to producing higher precocity and quantity of shoots, it enables higher phytosanitary control.

The micropropagation of the species presents numerous challenges, such as high fungal / bacterial contamination and oxidation. The use of biocides such NaOCl for a long period of time (2 % for 20 minutes) with PPM™ added to the culture medium shows a potential for disinfestation in the *in vitro* establishment. For *in vitro* multiplication, the usage of WPM and BAP (2 to 4 g L^{-1}) promotes higher shoot length, shoot number and leaf number. For indirect organogenesis by leaf segments, the cultivation with BAP and ANA was not responsive to the induction of callogenesis.

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