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PREGERMINATION TREATMENTS OF *TAXUS GLOBOSA* SEEDS: GROWTH AND *IN VITRO* EMBRYO CULTURE

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The Mexican yew (*Taxus globosa*) is important for the production of taxol, which is used to treat several types of cancer and its seeds undergo a latent period. The objectives of this research were to study the causes of seed dormancy in this species, the types of dormancy mechanisms it undergoes and how to break such dormancy. Seeds from two regions of Mexico (northern and central region) were evaluated using 12 pre-germination treatments. The treatments consisted of warm and cold stratification, applying 500 ppm of gibberellic acid ($GA_{4/7}$) at different intervals, or using alternative seed scarification methods. In addition, embryo growth was studied and a protocol for *in vitro* embryo culture was also tested. Seeds from the central region did not germinate, probably because of inbreeding depression in seeds that came from small fragmented populations. In contrast, seeds from the northern region of Mexico subjected to warm plus cold stratification, warm plus cold stratification plus $GA_{4/7}$ application, and warm stratification plus stratification with N2 plus $GA_{4/7}$ application exhibited the highest germination rates (12–14 %). *In vitro* embryo culture indicated that only 30% of the embryos germinated. Thus, dormancy in the Mexican Yew seems to be caused by several factors, including both morphological and physiological factors.

Keywords: Mexican yew, dormancy, embryo growth, embryo *in vitro* culture

INTRODUCTION

Taxus globosa is under special protection by the Mexican government (Secretaría de Medio Ambiente y Recursos Naturales, 2010). This tree produces a metabolite called taxol, which is used to treat different types of cancer (Shoeb 2006). The yew tree, as it is commonly known, is sporadically distributed, from the humid temperate regions of central Nuevo León and Tamaulipas, in Northeastern Mexico, to southern Honduras. There are few studies on this undomesticated species (Soto-Hernández et al. 2000, Zavala-Chávez 2001, Zavala-Chávez et al. 2001, Zavala-Chávez 2001, 2002, Soto-Hernández et al. 2011). This tree produces very small quantities of seeds, thus limiting its study and potential domestication. Germination difficulties in *T. globosa* was reported by Zavala-Chávez (2001) and Nicholson & Munn (2003). These studies refer to pregermination treatments that were relatively successful, but did not solve the problem of dormancy, a survival strategy in which seeds do not germinate even under favorable environmental conditions, but rather at different times, providing more opportunities for species survival in the wild, particularly under adverse environmental conditions.

There are several types of seed dormancy. However, seed dormancy in *T. globosa* has not been characterised. The embryo of this species is reported as immature or small (Ramírez-Sánchez et al. 2011). Nikolaeva (2001) classifies this condition as morphological dormancy. In other species of the same genus, presence of inhibitor substances are reported through a condition called physiological dormancy (Le Page-Degivry 1977, Chien et al. 1998).

Despite success in vegetative propagation of *T. globosa*, massive propagation with broad genetic diversity is necessary in restoration or genetic improvement programmes (Muñoz-Gutierrez et al. 2009). This is achieved through sexual reproduction, therefore, a high seed germination percentage, elimination of dormancy, as well as broad genetic variation in seedlings are desirable. It is necessary to define the mechanisms that cause seed dormancy to obtain rapid and homogenous germination. Nicholson & Munn (2003) applied pregermination treatments with relative success in *T. globosa*, and Ramírez-Sánchez et al. (2011) concluded that the seed coat permits imbibition. In *Taxus chinensis* var. *mairei* the problem of propagation has been approached

through *in vitro* culture, without obtaining favorable results (Liu et al. 2011). Chee (1994) cultured *in vitro* zygotic embryos of *T. brevifolia, T. baccata, T. baccata* var. *stricta* and *T. cuspidata* achieving germination percentages of 14, 14, 17 and 11% respectively. He indicated that seedling growth is faster with this method than by using natural embryo germination.

The objective of this research was to study diverse methods to break dormancy in *T. globosa* seeds, measure embryo growth, and fine-tune an *in vitro* propagation protocol for zygotic embryos of this species.

MATERIALS AND METHODS

Plant material

Taxus globosa fruits were collected in October 2012 in 11 localities, five in the state of Nuevo León, Mexico (northern region), and the other six in the states of Hidalgo and Querétaro, Mexico (central region) (Table 1). The trees of this species produce very small quantities of seed, and so the sample size used in the assessment of the germination variables was of 50 seed per repetition. The seeds were cleaned after harvesting and stored for two months under refrigeration at 5 °C before pregermination treatments started.

Viability, pre-germination treatments and germination tests

Before pregermination treatments, seed viability was determined using a 1% tetrazolium chloride test (International Seed Testing Association 2005). For each region, two replications with 25 seeds each were used. This test was repeated at the end of the experiment with seeds that did not germinate.

Pregerminative treatments were combined using warm and cold stratification, gibberellic acid and scarification methods, based on the work of Chien et al. (1998), Nicholson & Munn (2003) and Vance & Rudolf (2008). KNO_3 was included to stimulate germination, as suggested in agricultural crops and Pinaceae (International Seeds Testing Association 2005). The KNO_3 solution (0.5 % w/v) was applied twice a week to saturate the paper used as substrate. During warm stratification the seeds were being exposed to a photoperiod of 12 h with fluorescent light (736 µmol, m = 2, s = 1) and a thermoperiod of 15 °C at night and 23 °C during the day for five months. Cold stratification was set at 3 °C for two months in sphagnum. Gibberellic acid treatments involved submersing the seeds in 20 ml of a solution of 500-ppm $GA_{4/7}$ gibberellin for 24 h at room temperature. Three methods of seed scarification were applied: 1) immersion

Table 1 Geographic location of *Taxus globosa* populations sampled

Locality	Municipality, State	Latitude N	Longitude w	Elevation (m asl)
Northern region				
El Tejocote	Santiago, Nuevo León	25°19'20"	100°15'30"	1970
Cañada La Trinidad	Santiago, Nuevo León	25°14'49"	100°09'20"	1361
Yerbabuena	Zaragoza, Nuevo León	23°55'31"	99°47'58"	2100
La Encantada	Zaragoza, Nuevo León	23°55'20"	99°48'20"	2385
La Tinaja	Zaragoza, Nuevo León	23°53'25"	99°47'29"	2580
Central región				
Los Granadillos	Pinal de Amoles, Querétaro	21°12'40"	99°41'00"	2400
Cañada de Agua Fría	Pinal de Amoles, Querétaro	21°08'14"	99°41'06"	2570
Los Corrales	Mineral El Chico, Hidalgo	20°12'13"	98°43'33"	2550
Los Zorrillos	Mineral El Chico, Hidalgo	20°13'16"	98°43'08"	2400
Los Ayacahuites	Mineral El Chico, Hidalgo	20°12'25"	98°43'10"	2630
Pueblo Nuevo	Mineral El Chico, Hidalgo	20°11'04"	98°40'42"	2530

in 100 mL liquid nitrogen (N_2) and immediate extraction to room temperature, 2) immersion for 20 min in water at 70 °C and 3) immersion for 4 min in a concentrated solution of sulfuric acid (98% H_2SO_4).

The following treatments were applied: T_1 = control, T_2 = warm stratification, T_3 = warm stratification + cold stratification T_4 = warm stratification + application of $GA_{4/7}$, T_5 = warm stratification + cold stratification + application of $GA_{4/7}$, T_6 = warm stratification + scarification with N_2 + application of KNO_3, T_7 = warm stratification + scarification with N_2 + application of $GA_{4/7}$; T_8 = warm stratification + scarification with N_2, T_9 = warm stratification + scarification with water at 70 °C + application of $GA_{4/7}$, T_{10} = warm stratification + scarification with H_2O at 70 °C T_{11} = warm stratification + scarification with H_2SO_4 + cold stratification + application of $GA_{4/7}$ T_{12} = cold stratification and T_{13} = warm stratification + application of KNO_3.

Four replications with 50 seeds each were used for each treatment. Before applying the treatments, the seeds were washed and disinfected by submersion in a solution of 1.5% sodium hypochlorite and 25% ethanol for 15 min. They were then rinsed with distilled water. Each lot of previously disinfected seeds was placed on moist paper in plastic containers (4 L in volume) and transferred to a germination chamber with photoperiod and temperature regime similar to that of warm stratification. The treatments started at different times depending on the time needed to complete pre-germination treatment. The longest pre-germination treatments (T_3, T_5 & T_{11}) lasted seven months. Then, the seeds were placed in the germination chamber. The germination process began at the same time and lasted six months. The entire experiment lasted 13 months. Seeds are considered germinated when the radicle reache a length of 2 mm (Chien et al. 1998). Once germination is initiated, the number of germinated seeds were counted every week. Germination percentage was determined at the end of the test. Germination rate (GR) was determined using the Maguire (1962) index:

$$GR = \Sigma Xi/Ni$$

where Xi = number of germinated seeds at week Ni, and Ni = number of weeks after sown.

Mean time to maximum germination (MT) was calculated as proposed by Edmond & Drapala (1958):

$$MT = (\Sigma XiTi)/(\Sigma Xi)$$

where Xi = number of germinated seeds per week and Ti = time.

Embryo growth

At the beginning of the pregermination treatments and three months after starting the germination test, a sample of 25 seeds was taken, each seed selected at random, to assess embryo growth using non-destructive image analysis. Excess water was eliminated to obtain sharper images as embryos passed through a Faxitron X-ray V.30. One radiograph was taken per treatment with an exposure of 26 kV for 3 s. Embryo length was measured using a software accompanying the X-ray equipment. Embryo images in treatments where germination was obtained at the end of the experiment (T_3, T_4, T_5, T_6 and T_7) and control (T_1) were analysed. Additional observations were made on seeds dissected under a Leila stereoscopic microscope.

In vitro embryo culture

Since the seed collection from the northern region was more abundant, 320 seeds from this region were separated into two groups (control and cold stratified). Within these two groups, four dosages of GA_3 were tested: 0 (control), 1, 100 and 500 ppm. There were 40 seeds per treatment in a 2 × 4 factorial design. The seeds were disinfected with a fungicide solution (0.2 g L^{-1}) and a bactericide (0.6 g L^{-1}) for 24 h, rinsed with distilled water, placed in 3% sodium hypochlorite for 10 min and washed three times with sterile water. The control (non-stratified) group was kept at room temperature (21–22 °C), while the cold stratified group was kept at 3 °C for 48 h and at 21 °C for 1 day. All of the seeds were then dissected to extract the embryos, which were sown in solid MS medium with the different dosages of GA_3. Temperature was 25 ± 2 °C and 12 h of light. Ten embryos were placed in each recipient (experimental unit), and there were four replicates (40 seeds) per treatment. Percentage of germinated embryos per experimental unit was calculated. Embryos were considered germinated when they reached a length of 1 mm and exhibited green coloring. Measurements initiated a week after seeding in MS medium and continued for 30 days.

Statistical analysis

To evaluate the effect of the pregermination treatments on germination traits, a one-way analysis of variance was performed since the seed from one of the geographic regions did not germinate. The statistical analysis was done using PROC GLM (SAS Institute 1998).

An analysis of variance was performed with PROC ANOVA to determine whether there were significant differences in embryo length among treatments (SAS Institute 1998), considering each embryo as a replicate. In this analysis, only those treatments in which seeds germinated were included. To determine embryo growth, the data on embryo length from the images taken on the two sampling dates (initial and three months after starting the germination test) were used.

Regarding *in vitro* embryo culture, the statistical analysis used to assess the effect of the treatments on germination percentage was a 2 × 4 factorial arrangement in a completely random design with four replications.

Germination percentage did not adjust to a normal distribution, so prior to analysis, data was transformed with the arsin [sqrt(x)] to improve its normality (Sokal & Rohlf 2012). In the cases where significant differences ($p \leq 0.05$) were found among treatments, the Tukey comparison of means test ($p \leq 0.05$) was used.

RESULTS AND DISCUSSION

Viability, pregermination treatments and germination

Seed viability was 98% for the northern region and 60% for the central region, and no empty seeds were found. Even though the germination test lasted six months and all the sampled seeds contained embryos, none of the seeds from the central region germinated. This could be associated with a high level of inbreeding, a common phenomenon in small, isolated populations, as those sampled in this region. In other Mexican tree species with small and fragmented populations, such as *Pseudotsuga menziesii*, low levels of genetic diversity have been found (Cruz-Nicolás et al. 2008) as well as smaller or empty seeds, low germination percentages and higher frequency of albino or mutant seedlings, in contrast with the larger populations of the northern region of the country (Juárez-Agis et al. 2006; Mápula-Larreta et al. 2007). Ramírez-Sánchez et al. (2011) conducted a study on the same seed lots used for the research and determined that *T. globosa* seeds from the central region are 28% smaller than those of the northern region. Inbreeding might also affect embryo development and depth of dormancy, making it necessary to use a different treatment from those applied in our study. However, to validate this hypothesis, it would be required to conduct additional genetic studies. On the other hand, the lack of germination may be due to unsuitable treatments for seeds from populations of the central region, so different environmental conditions (photoperiod, temperature, etc.) or a longer incubation period may be required. García-Aranda et al. (2011) mentioned that *T. globosa* populations in the northern region belong to the variety *floridana*. This may indicate different ecotypical behavior, with adaptation to different environmental requirements for optimal seed germination. These adaptive differences point the need to study seed development and germination specifically in this geographic region.

Germination observed in the control treatment of the northern region (Figure 1) shows how the embryo begins breaking the morphological and physiological barriers over a relatively long time (> 8 months). When seeds are collected, they appeare to be mature, exhibiting a red aril, however, this does not necessarily reveal the seed's physical maturity and germination capacity (Ramírez-Sánchez et al. 2011). Despite the low germination proportion, it might be enough for long-term survival of the species in its natural habitat. A staggered germination grants the species opportunities for survival over time by forming a seed bank in the soil. The seeds remain until conditions are optimum for germination and seedling establishment (Nikolaeva 2001).

A significant higher germination percentage was found when treatment T_3 was applied to seed from the northern region (Figure 1), as compared to the other treatments, although no significant statistical differences were found between T_3, T_5 and T_7.

These results indicate that warm stratification + cold stratification (T_3 and T_5) might have promoted maturation of the embryos. The embryos grew large enough for germination and possibly physiological dormancy was broken (Le Page-Degivry 1973, Milhorst 2007). Ramírez-

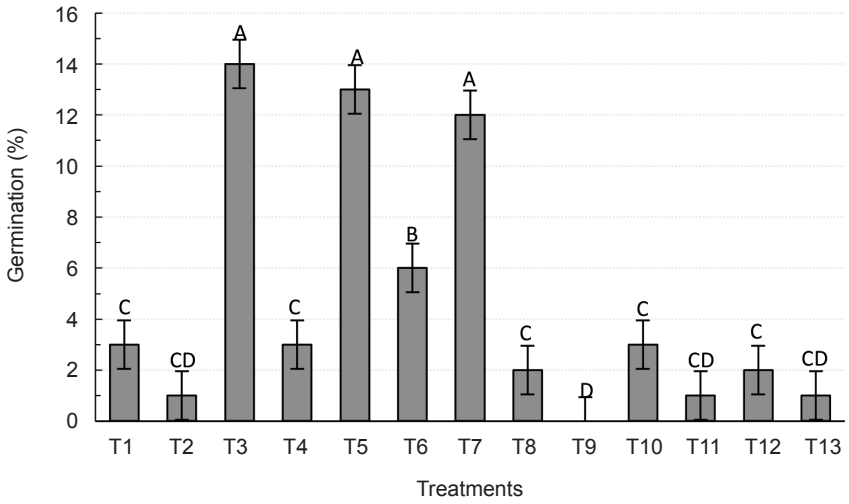

Figure 1 Germination percentage of *Taxus globosa* seeds subjected to different pre-germination treatments; vertical bars represent standard error (SE), means with different letters are significantly different (Tukey, $p \leq 0.05$)

Sánchez et al. (2011) reported that in recently collected seed, the proportion of embryo to total volume of seed varies between 3 and 4%, and average embryo length is 1.92 mm for seed from the northern region and 1.72 mm for seed from the central region. These values were smaller than those reported for other *Taxus* species. According to Forbis et al. (2002), the embryo occupies 10% of the seed volume on average in Taxaceae. The small size of *Taxus globosa* embryos would limit germination capacity of most seed, but the warm stratification period allows the embryo to develop to a stage at which it could germinate. In addition, growth inhibitors are reported to be present in the seed of other *Taxus* species (Le Page-Degivry 1973, Chien et al. 1998). Although our study did not include detection of these substances, it is expected that some of the treatments in which cold stratification was applied would reduce its concentration. Chien et al. (1998) observed a decrease from 8888 to 536 picogram per seed in the concentration of abscisic acid after subjecting *Taxus mairei* seeds to cold stratification at 5 °C for 12 months.

Based on results from the literature, the pre-germination treatments were combined to evaluate their response. For this reason, some treatments, such as T_5 were included. This treatment resulted in a germination percentage (13%) similar to that from T_7 (12%) (Figure 1). It was expected that the addition of gibberellin would raise the germination percentage, but that did not happen, since T_3 resulted in 14% germination. For this reason, the possibility of applying GA before cold stratification was explored, in order to expose the seed to GA for a longer time. As for T_7, it is possible that scarification with liquid nitrogen may have caused ruptures in the cell membrane of the seed coat (García-de los Santos & Steiner 2003), so when GA was added, seed germination was promoted.

Regarding warm stratification time, it is possible that a longer period is necessary to achieve higher germination capacity. Other studies have obtained higher germination percentages with longer incubation times. Chien et al. (1998) achieved 81% germination in *T. mairei* after six months of exposing seeds to warm stratification plus three months of cold stratification. Nicholson & Munn (2003) reported 34% germination in a sample of 50 *T. globosa* seeds after nine months of warm stratification using alternating temperatures, although they did not provide precise details of their experimental methods.

Regarding the germination rate index (Figure 2), the highest values obtained were parallel to the high germination percentages, which had significant differences ($p \leq 0.05$) when compared to the other treatments. The mean time needed to reach maximum germination was 266 days for T_3, 220 days for T_5 and 250 days for T_7, but there were no significant statistical differences among them. These treatments, however, were superior ($p \leq 0.05$) to the other treatments. Two of them,

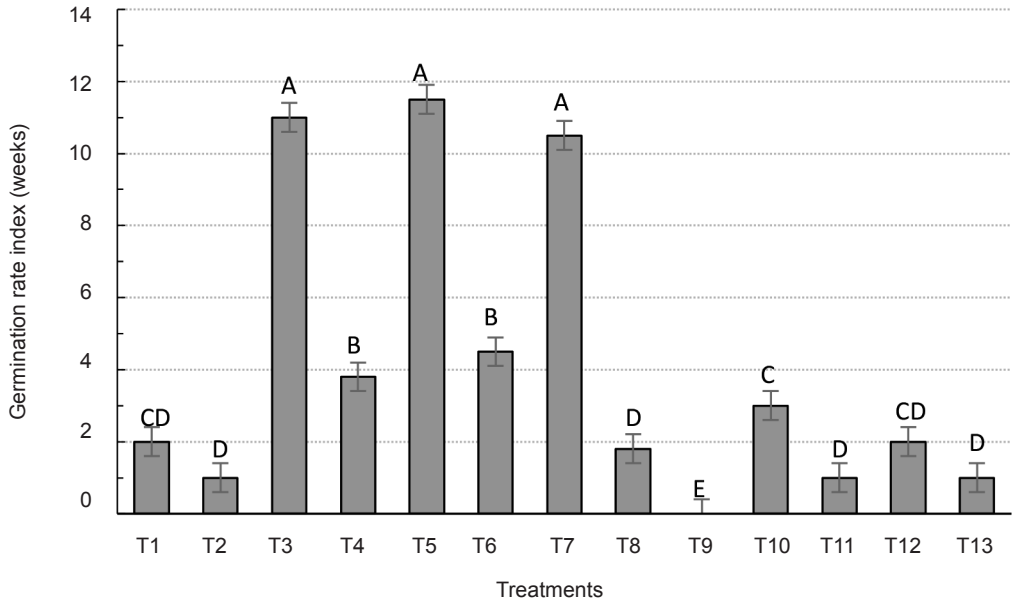

Figure 2 Germination rate index of *Taxus globosa* seeds subjected to different pregermination treatments; vertical bars represent standard error, means with different letters are significantly different (Tukey, $p \leq 0.05$)

T_5 and T_7, included exogenous application of GA to promote germination, but high germination percentages were not obtained.

Embryo growth

Significant differences ($p \leq 0.05$) in embryo growth were found among warm stratification treatments promoting seed germination. Seeds in T_3, T_4 and T_7 achieved the greatest embryo length (Figure 3 and 4). At the beginning, embryo length was 1.6 mm, while in these treatments average embryo length was of 4.4 mm. In T_3 and T_7, embryo growth due to warm stratification treatments would partially explain the higher germination percentage at the end of the germination period in these treatments.

Embryo growth in the control treatment was the lowest. Warm stratification was important for initiating embryo development since the embryo is small and protective layers impede its development, thus, growth is relatively slow. According to Baskin & Baskin (2004), seeds that have underdeveloped embryo possess morphological dormancy, therefore cannot germinate. *Taxus globosa* seed, however, besides morphological dormancy, appears to have physiological dormancy.

Therefore, it is possible to classify *T. globosa* seed as having morpho-physiological dormancy. Here, besides the underdeveloped embryo, a physiological inhibitor, such as abscisic acid (ABA), seems to be causing dormancy. Thus, *T. globosa* seed requires warm stratification (the period in which the embryo develops) plus cold stratification with application of GA to promote germination. Nikolaeva (2001) refers to this type as endogenous dormancy and deals with depth of dormancy. Non-germinated seed were viable at the end of germination period, so they might require longer cold stratification.

In vitro embryo culture

Embryo germination in culture medium was observed after two weeks and continued for up to 30 days after seeding. A significant effect of cold stratification was not observed. There was, however, response ($p \leq 0.05$) to the application of GA (Table 2), although the three dosages of GA_3 used produced statistically similar responses, with the exception of 100 ppm GA_3 without cold stratification.

In this study, the effect of adding $GA_{4/7}$ and GA_3 at different stages of the germination trial was assessed. It was found that the two types of GA had favorable effects on embryo germination.

Figure 3 Dissected seeds showing embryos in T_3 (warm stratification + cold stratification), A = beginning of pre-germination treatment (1.6 mm, 0 d), B = after 180 d and C = after 270 d (4.4 mm in average length)

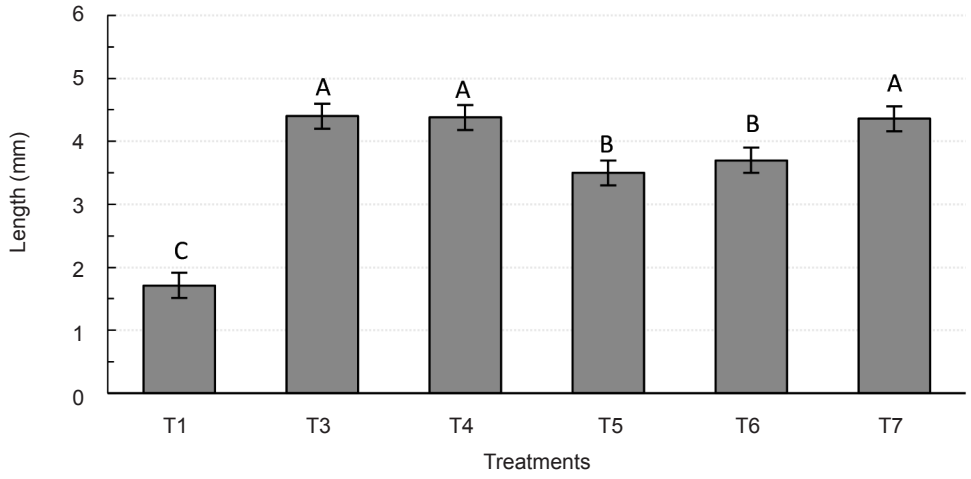

Figure 4 Average embryo length after five months of warm stratification in those treatments achieving higher seed germination (T_3–T_7), control (T_1) is showed for comparison; vertical bars represent standard error (SE), means with different letters are significantly different (Tukey, $p \leq 0.05$)

Table 2 Germination percentage in *in vitro*-cultured *Taxus globosa* Embryos, cold stratified (+) and non-stratified (-)

Variable	No GA_3		1 ppm GA_3		50 ppm GA_3		100 ppm GA_3	
	+	-	+	-	+	-	+	-
Germination (%)	0	0	30.0 a	25.0 ab	17.5 ab	12.5 ab	15.0 ab	7.5 b
Standard error	0	0	0.408	0.866	0.479	0.629	0.866	0.479

Average values within a row with different letters are statistically different ($p \leq 0.05$), GA = gibberellic acid

The results of this trial should provide direction for further research with both GA types in order to standardise a method for enhancing seed germination, be it *in vitro* or normal culture. In the case of *T. baccata* GA was not required for *in vitro* culture of zygotic embryos; the MS medium was enough to achieve embryo germination *in vitro* (Zarek 2007, Hosseini et al. 2011), unlike the findings of our study in which gibberellin was needed.

Studies with other *Taxus* species have found that *in vitro* culture solves the problem of dormancy since they seem to have an impermeable seed coat (Chien et al. 1998, Zarek 2007, Hosseini et al. 2011). This is not the case of *T. globosa;* when the seed coat and the megagametophyte were removed, some *in vitro* cultured embryos germinated. Although this indicated that in these organs lies a mechanism causing seed dormancy, only 30% of the embryos germinated.

Finally, regarding geographic seed origin (Northeastern and Central Mexico), continued research is needed on the current genetic status of populations, particularly those in the central region. The fact that no germination was achieved in seeds collected in these populations, is an indicator of low reproductive success, which might threatens their existence in natural habitats.

CONCLUSIONS

The seed of *T. globosa* has an underdeveloped embryo; warm stratification for five months promotes embryo growth. *Taxus globosa* seed has a complex dormancy, characterising it as morpho-physiological dormancy. When the seed coat and the megagametophyte were removed, *in vitro* embryo germination was achieved. These organs may form part of the mechanism causing dormancy. No germination was accomplished with seeds from central Mexico.

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