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# In Vitro Germination Of Picea Chihuahuana In Different Culture Media And Light Conditions

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***Abstract*— *Picea chihuahuana* Martínez is endemic to Mexico and mainly restricted to the northern part of the state of Durango. The species is listed as at risk of extinction (NOM-059-SEMARNAT-2010). Of the biotechnological and tissue culture techniques available, somatic embryogenesis and organogenesis are viable alternatives to in situ/circa situm conservation processes and/or use of natural populations. In the present study, we compared the performance of five culture media (MS, QL, B5, SH and WPM) in relation to germination of *Picea chihuahuana* Martínez seeds. A protocol was developed for the disinfection and dissection of the seeds before sowing in the culture media. Seeds were cultured under two controlled conditions in germination chambers (26±2 ºC and darkness, 0 LX) and (26±2 ºC and 16 h photoperiod at 1052 LX) for 28 days. The MS medium yielded the highest germination rate in darkness (29.75 %), and QL yielded the highest rate under the 16 h photoperiod (53.25 %). The QL medium under the 16h photoperiod yielded the highest germination, which was significantly higher than yielded by medium B5.**

***Keywords*— *Picea chihuahuana; tissue culture; in vitro nutrition; germination behaviour.***

## I. INTRODUCTION

The forests in the state of Durango are of enormous potential in relation to conservation of the environment and sustainable improvement of living conditions. However, the forests are subjected to disturbance, mainly as a result of human activity, leading to substantial reductions in the number of living trees [4] as in the case of *Picea chihuahuana*. The current poor conservation status of this species is due to natural and anthropogenic fragmentation of populations, irregular reproduction processes and illegal forest harvesting [1]. For massive propagation of *Picea chihuahuana*, the growth requirements of the species must be determined and propagation methods that will be available for other economically, ecologically and socially important forest species must be developed. This will help recover populations in sites where trees have declined or completely disappeared.

The geographical distribution of the Chihuahua spruce *Picea chihuahuana* Martínez is restricted to the northern part of the Sierra Madre Occidental (SMO). The main sites of distribution within the state Durango are the municipalities of Pueblo Nuevo, Tepehuanes and Guanaceví [5]. *Picea chihuahuana* populations are relicts. The trees have a characteristic conical crown and usually grow to heights of between 25 and 35 m, with the stem diameter reaching 90 cm at a height of 1.30 m [5]. In an ecological study of *Picea chihuahuana* in the south of the state of Durango, Gordon [6] found that the populations varied from between 5 and hundreds of individuals that were naturally associated with other conifers and oaks and often with *Cupressus* spp., *Pseudotsuga* spp., *Abies durangensis*, *Pinus ayacahuite*, *Alnus* spp. and occasionally with *Populus tremuloides*. *Picea chihuahuana* is the only species of the genus known within the state of Durango; it is listed in the Official Mexican Standard (059) and is considered an endemic relict [4, 15].

*In vitro* culture techniques have become increasingly important in recent years, complementing conventional conservation methods [17]. The success of *in vitro* establishment and growth of plant tissue is generally determined by the nature of the explant, the composition of the nutrient medium and various environmental factors, such as light and temperature [3]. The *in vitro* propagation of forest species has been well studied, and various modern techniques that may eventually substitute traditional plant propagation methods have been developed [10]. In recent years, numerous studies have successfully applied *in vitro* propagation techniques to conifer species such as *Pinus carinaea*, *Pinus maximartinezii* [16], *Fitzroya cupressoides* and *Picea glauca* [21]. *In vitro* germination and growth techniques are useful and provide certain benefits for plant regeneration as they are carried out under controlled, aseptic conditions [8, 10,]. At present, *in vitro* propagation of forest species

is mainly carried out by organogenesis and embryogenesis. Although still at a relatively early stage of development, these techniques sometimes even substitute conventional plant propagation methods [10, 22]. In conifers, somatic organogenesis (clonal propagation) is the most commonly used technique and consists of developing buds or root meristems from explants obtained directly from the individual tree of interest or from a set of undifferentiated cells [18].

Although the technology is available, no protocol for *in vitro* micropropagation of *Picea chihuahuana* plantlets has yet been developed. The aim of the present study was to compare the performance of five different culture media for the *in vitro* germination of the species before determining the nutrient contents of the shoots to establish the optimal basal medium for production.

## II. Materials and Methods

The study was carried out at the Laboratory of Genetics and Plant Production in the Institute of Silviculture and Wood Industry, University Juárez del Estado de Durango. The seed was obtained from the Ejido Chiqueros y Anexos in the municipality of Guanaceví, Durango (26º 08´41.07´´ W, 106º 22´ 54.27´´ N, elevation 2,762 masl).

### *A. Culture media preparation*

Five culture media were prepared using the nutrient concentrations proposed by Sigma-Aldrich [20] (Table I): 1) B5 medium (Gamborg, Miller and Ojima, 1968); 2) QL medium (Quoirin and Lepolvre, 1981); 3) WPM (woody plant medium) (Lloyd and McCown, 1980); 4) MS (Murashige and Skoog, 1962); and 5) SH (Schenk and Hildebrandt, 1972). Aliquots of 20 $cm^3$ of each basal medium were prepared in glass flasks of volume 120 $cm^3$.

TABLE I. Composition of Sigma-Aldrich culture media (2007)

| Components | Culture medium [mg/L] | | | | |
|---|---|---|---|---|---|
| | ***B5*[1]** | ***QL*[2]** | ***WPM*[3]** | ***MS*[4]** | ***SH*[5]** |
| Ammonium nitrate | 0 | 400.00 | 400.00 | 1850.00 | 300.00 |
| Monobasic ammonium phosphate | 34.00 | 0 | 0 | 0 | 0 |
| Ammonium sulphate | 1.00 | 0 | 0 | 0 | 0 |
| Boric acid | 3.00 | 6.20 | 6.20 | 6.20 | 5.00 |
| Calcium chloride | 113.24 | 0 | 72.50 | 332.20 | 151.00 |
| Calcium nitrate | 0 | 833.77 | 386.00 | 0 | 0 |
| Cobalt chloride - $6H_20$ | 0.02 | 0.02 | 0 | 0.02 | 0.10 |
| Copper sulphate - $5H_20$ | 0.02 | 0.02 | 0.25 | 0.02 | 0.20 |
| Disodium EDTA | 37.30 | 37.30 | 37.30 | 37.26 | 20.00 |
| Ferrous sulphate - $7H_20$ | 27.80 | 27.80 | 0 | 27.80 | 15.00 |
| Manganese sulphate | 122.09 | 175.79 | 180.70 | 180.70 | 195.40 |
| Magnesium sulsphate - $H_20$ | 10.00 | 0.76 | 22.30 | 16.90 | 10.00 |
| Ammonium molybdate - $2H_2O$ | 2500.00 | 1800.00 | 0 | 1900.00 | 2500.00 |
| Potassium iodide | 0 | 270.00 | 170.00 | 170.00 | 0 |
| Potassium nitrate | 990.00 | 0 | 0 | 0 | 0 |
| Monobasic potassium phosphate | 130.50 | 0 | 0 | 0 | 0 |
| Potassium sulphate | 2.00 | 8.60 | 8.60 | 8.60 | 1.00 |
| Monobasic sodium phosphate | 2500.0 | 1800.0 | 0 | 1900.0 | 2500.0 |
| Zinc sulphate - $7H_2O$ | 0 | 270.00 | 170.00 | 170.00 | 0 |

### *B. Disinfection and sowing*

The germplasm was disinfected following the protocol proposed by Mroginski [12], for posterior sowing under isolated conditions under a laminar flow cabinet.

The seed coat was completely removed with the aid of tweezers and scalpel, and care was taken to prevent damaging the cotyledons and the embryo. The seed was then placed on the basal medium, in the center of the flask. The same procedure was used for sowing all five basal media evaluated.

The flasks were transferred to growth chambers at a constant temperature of 26±2 ºC and one of the following two lighting regimes, for 28 days: 1) photoperiod of 1052 LX for 16 h and darkness for 8 h, and 2) complete darkness (0 LX).

### *C. Information analysis*

Two experimental runs consisting of three replicates of each of 33 experimental units were evaluated. After sowing the seeds, the number of plantlets that developed was monitored and the cumulative germination was recorded every 24 hours for 28 days. The data obtained were subjected to factorial analysis in which factor A was the light regime (two levels: darkness, 0 LX, and 16 h photoperiod, 1052 LX) and factor B was the culture medium (five levels: B5, QL, WPM, MS and SH). The mean values obtained were analyzed by Tukey`s test (α=0.05), implemented with SAS Ver. 9.1 [19].

## III. Results and Discussion

The statistical analysis revealed that under conditions of darkness (0 LX), germination of the *P. chihuahuana* seedlings was highest (29.75 %) on MS culture medium and lowest (6 %) on medium B5. Under the 16 photoperiod (1052 LX), medium QL yielded the highest germination rate (53.25 %), and medium B5 yielded a germination rate of 7.25 % (Figure I). The five culture media under study had different effects on the germination of *P. chihuahuana*.

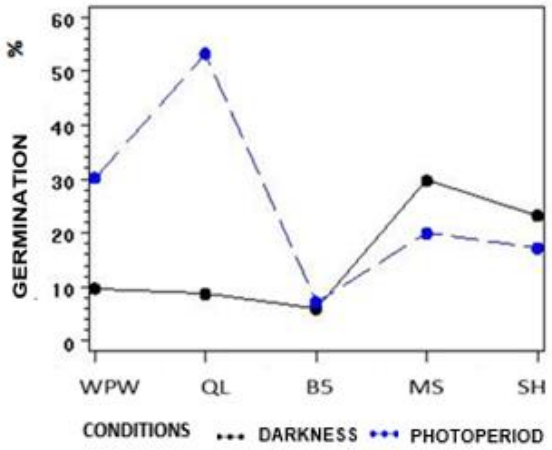


FIGURE I. CUMULATIVE GERMINATION IN *PICEA CHIHUAHUANA* UNDER DIFFERENT LIGHT CONDITIONS

Gamborg [3] probed the elaboration of different culture media, which were assigned different numbers. Medium B5 was formulated for culturing soya callus, but is not particularly recommended for *Picea chihuahuana* as it yielded the lowest germination response under both light conditions (Figure II). The light conditions are important as germination of seedlings on the basal media generally responded favorably to the presence of light, except with MS and SH, which yielded better results in darkness (in Figure. II, the number 1 on the x axis represents darkness and number 2 represents the 16h photoperiod).

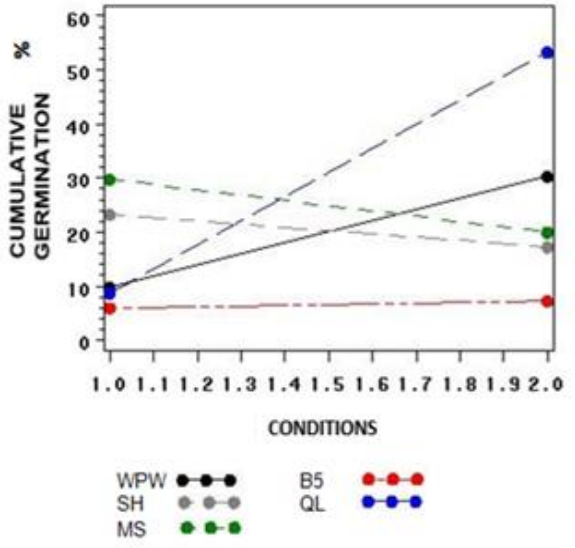


FIGURE II. GERMINATION CONDITIONS

Comparison of the mean values by the Tukey test revealed three different groups; group A included QL and MS; group AB included SH and WPM, and group B included medium B5 (Table II).

TABLE II. COMPARISON OF MEAN NUMBER OF GERMINATED INDIVIDUALS ON DIFFERENT CULTURE MEDIA*

| Culture medium | Mean number of individuals germinated |
|---|---|
| QL | 31.00 A |
| MS | 24.87 A |
| SH | 20.25 BA |
| WPW | 20.00 BA |
| B5 | 6.62 B |

**Mean values indicated by the same letter are not significantly different (α=0.05).*

The interaction between light conditions and the culture medium was also evaluated; the interaction with the most positive effect on the germination of *P. chihuahuana* seeds was that between the QL and the 16h photoperiod (interaction 7), which yielded a germination rate 53.25 % (Table III). This is consistent with the findings of other studies reporting successful in vitro germination of *Pinus radiata* D. Don seedlings in QL medium [11]. The germination rate was significantly higher than that obtained on WPM, which was specifically designed for culturing tissues of woody plants and which is used for propagation and root elongation of some species (Franco et al., 2006). The results obtained in the present study contrast with those reported by Correidoira [2], who obtained favorable results with WPM and by applying axillary root proliferation protocols for the micropropagation of woody species and trees in Spain.

TABLE III. CUMULATIVE GERMINATION IN *PICEA CHIHUAHUANA* ACCORDING TO INTERACTION

| Condition | Medium | Cumulative germination (%) | Interaction |
|---|---|---|---|
| Darkness | WPW | 9.75 | 1 |
| Darkness | QL | 8.75 | 2 |
| Darkness | B5 | 6.00 | 3 |
| Darkness | MS | 29.75 | 4 |
| Darkness | SH | 23.25 | 5 |
| 16/8 h light/dark | WPW | 30.25 | 6 |
| 16/8 h light/dark | QL | 53.25 | 7 |
| 16/8 h light/dark | B5 | 7.25 | 8 |
| 16/8 h light/dark | MS | 20.00 | 9 |
| 16/8 h light/dark | SH | 17.25 | 10 |

This may be attributable to the concentration of the macronutrients administered, as nitrogen, phosphorus and potassium represent more than 75% of the mineral nutrients found in plants.

The formulation of the basal medium used depends on the aims and which particular tissue culture techniques are applied, as well as the species or type of plant being propagated. The basic nutritional requirements of the cultivated plant material are similar to those of material planted in the field. The Schenk and Hiledebrandt (SH), Gamborg (B5) and Murashige

and Skoog (MS) media contain large amounts of macronutrients, whereas the others contain fewer inorganic compounds.

The light conditions had significant effects on the germination of *P. chihuahuana* seeds. This has also been found in other woody and non-woody species, and it has been suggested that the age of the seed, as well as the degree of maturity and alleopathic effects should also be considered [14],

However, the germination rates obtained in the present study were low compared with those of other species, as *Picea chihuahuana* is naturally somewhat difficult to regenerate and also because of other problems encountered in its natural habitat [6].

## IV. Conclusions

Germination of *P. chihuahuana* seeds was under the 16 h photoperiod was maximal on medium QL and in darkness on MS medium. We can assume from the nutrient composition of the culture media that the presence of ammonium nitrate was essential for the germination response in *P. chihuahuana*. The presence of compounds such as monobasic ammonium phosphate, ammonium sulphate, potassium sulphate and monobasic sodium phosphate had inhibitory effects on the germination of this species.

Under the conditions used in the present study, the culture medium that yielded the best results in terms of germination and maintenance of *P. chihuahuana* plantlets was QL under the 16h photoperiod. This study contributes to improving the initial method for generating shoots and the massive reproduction in vitro of *P. chihuahuana*, as a viable option for propagating and conserving the species. Use of this culture medium may enhance the efficiency of germination and propagation; however, spectral analysis of the shoots generated is recommended in order to identify the nutrient components that significantly affect the germination process.

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