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# Behavior and preservation of an *in vitro* collection of European aspen in Spain

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**Key words**: *Populus tremula*, germplasm conservation, *in vitro* plant culture, micropropagation, selected clone.

**ABSTRACT:** An *in vitro* collection has been established with selected European aspen from Palencia province (Spain). Currently, this collection includes 32 high quality clones, selected for their good bearing and healthy state. Most of them belong to different discrete local populations.

*Populus tremula* L. was propagated in proliferation Aspen Culture Medium; they required subculture every 3 months. The purpose of this study was, therefore, to select a medium which allows the maintenance of 32 clones for a period longer than 3 months without subculture and to observe the behavior of those clones in 15 different culture medium compositions. Seven nodal cutting stem explants from each clone were cultured in parallel in the different media. One and three months after setting on the stem explants, the number and the size of shoots, the root size, the presence or absence of callus and the survival, were evaluated. The survival was monthly recorded during 8 months. Taking into account the explant development, four media were proposed for collection preservation. One of them, Ga, with a reduction of salts, sucrose, 6-benzoaminopurine, omitting adenine sulphate and 1-naphthalene acetic acid, is the most economical. Behavior observations of the 32 clones in the 15 medium compositions showed the influence of the genotype of the clones.

**Abbreviations:** BA: 6-benzoaminopurine, NAA: 1-naphthalene acetic acid, ACM: Aspen Culture Medium, MS: Murashige & Skoog

## Introduction

Demand for germplasm preservation of various plant species has recently increased due to pollution, climate change, natural destruction and human impact on the ecosystem biodiversity (Son *et al*., 1997). Worldwide area of total forest extension is rapidly declining (Singh and Janz, 1995). As a result, there has been a decrease in the genetic pool of forest tree species. An attempt to preserve the biodiversity of natural stand of *Populus tremula* L. from the south-western limit of it Eurasia distribution allowed to inventory and select clones from each location and to initiate an *in vitro* collection. The *P. tremula* stand in the Central-North of Spain occurs in scattered patchy population beside small streambeds in mixed broad-leaved forest or in population along rivers. Theses populations are composed of a small number of individuals often less than a hundred

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covering around one hectare with a genetic diversity higher than expected (Lopez-de-Heredia *et al*., 2004).

Seed storage is a common strategy for germplasm preservation of most plant species. For *Populus* species, seed survival under natural conditions is short. Even then, seed propagation of selected tree genotypes may not guarantee the preservation of all the genetic traits. *In situ* and *ex situ* conservation of vegetatively propagated plants is a challenge due to the vast extension requirement and the difficulty in controlling pests and diseases. Nowadays the preservation of clonal germplasm is oriented on cryogenic techniques. Long term preservation of transgenic germplasm trees and natural hybrids are used for gene expression studies and other researches (Wullschleger *et al*., 2002; Tsai and Hubscher, 2004). *In vitro* preservation is another option. *In vitro* culture is advantageous for different reasons a) entire sets of genetic materials can be copied through regeneration schemes, b) rapid proliferation is achievable, c) disease-free plants can be produced and maintained, minimal space is required, d) risks related to environmental changes are avoided and f) behavior studies of clones in different medium compositions. This characterization gave useful information about the selected clones.

Within the framework of projects dedicated to the study of the natural population of *P. tremula* from Palencia (Spain), continuous productions of plants are required. The conservation studies, productive characterization among other reasons made necessary the establishment of an *in vitro* collection of 32 selected clones (Martínez-Zurimendi *et al*., 2003). Several reports of *in vitro* propagation technologies of poplars have been published (Ahuja, 1983; 1987; Chen and Huang, 1980; Park and Son, 1988; Douglas, 1985; Jokipii *et al.,* 2004). Plantlets were regenerated from shoot tip, nodal stem and other tissue and organ cultures, in many clones with a high regeneration capacity. Many clones were used for large-scale production and their yield was found superior to conventionally propagated plants.

Initially the *P. tremula* collection was subcultured in the Aspen Culture proliferation Medium (ACM) described by Ahuja (1983). To maintain the clones in good conditions, subculture is needed every 3 months, which is laborious and requires a lot of basic materials. Starting from the medium Murashige and Skoog (MS) (1962) many different plants were micropropagated *in vitro*, using adapted media. With regard to MS, the ACM has lower concentrations of $NH4^+$ and $K^+$ but maintains the same microelements and vitamins composition. These

**TABLE 1.**

**Composition of the different media tested**
**Medium A is the ACM medium described by Ahuja (1983). Macronutrient compositions were described in ACM, micronutrient and vitamin compositions were described by Murashige and Skoog (1962).**

| Media | A | B | C | D | E | C' | Ca | Cb | Cd | G | Ga | Gb | Gd | 1/2G | 1/4G |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Macronutrients A (10x) (ml of stock solution) | 100 | **50** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | **50** | **50** | **50** | **50** | **25** | **12.5** |
| Macronutrients B (10x) (ml of stock solution) | 100 | **50** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | **50** | **50** | **50** | **50** | **25** | **12.5** |
| Micronutrients MS (1000x) (ml of stock solution) | 1 | **0.5** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | **0.5** | **0.5** | **0.5** | **0.5** | **0.25** | **0.125** |
| Fe-sequestrene (mg) | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 |
| Agar (g) | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Vitamins (100x) (ml of stock solution) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Sucrose (g/l) | 20 | **13,3** | **13,3** | **13,3** | **13,3** | **10** | **10** | **10** | **10** | **6.7** | **6.7** | **6.7** | **6.7** | **3.3** | **1.6** |
| Adenine sulphate (mg/l) | 20 | **10** | 20 | | | **10** | | | **10** | **5** | | | **5** | **2.5** | **1.25** |
| NAA (mg/l) | 0.015 | 0.015 | | **0.2** | | | | | | | | | | | |
| BA (mg/l) | 0.5 | 0.5 | **0.2** | **0.2** | **0.2** | **0.1** | **0.1** | | | **0.05** | **0.05** | | | **0.025** | **0.0125** |
| Activated charcoal (g/l) | | | | | 5 | | | | | | | | | | |

Number indicate quantities of each constituent for 1 litter of (1x) medium

nutrients are essential for growth, organogenesis, enzymatic system activity and regulation. Sugar acts as a source of carbon, energy and has also an osmotic role. The high induction of callus and shoots is due to growth regulators like auxins, cytokinins and adenine sulphate (Krikorian *et al*., 1990). Moreover, *in vitro* micropropagated plants developing callus can lead to somaclonal variations. Variation happened more frequently in poplars of the *Leuce* section (8%) than in those of *Aigeiros* or *Tacamahaca* sections (1%) (Antonetti and Pinon, 1993). However, tissue culture promoting genetic alteration, which is undesirable for germplasm preservation.

The first purpose of this study was to select the medium that allow the maintenance of the whole collection (of 32 clones) in optimum conditions for many time, with few subculture. These conditions may contribute to reduce the cost and the number of manipulations.

## Materials and Methods

### *Plant material*

The 32 clones were maintained *in vitro* for 2-3 years in Aspen Culture Medium (ACM). Regular inventories of the collection showed that 20-25% of the subculture was changed every month. The selected clones were freshly cultured in ACM medium, during two months, after clone growth were tested in 15 media, were proliferating and rooting. They growth in culture chamber with consensus temperature, humidity and light and dark hour's cycle were used: 22ºC, 80% relative humidity and a 16:8 photoperiod.

### *Plant culture media*

ACM was modified as described in Table 1, and 14 other media were prepared. **Medium A is the ACM** proliferation medium described by Ahuja (1983). The main modifications consisted on the reduction of strength medium, sucrose and growth regulators concentrations. The macronutrients (A and B) stocks solutions were 10x concentrated, the micronutrient (MS) stock solution was 1000x concentrated and the vitamin solution was 100x concentrated. Chemicals and salts were purchased from Merck, adenine sulphate, 1-naphtalene acetic acid (NAA), 6-benzylaminopurine (BA), activated charcoal and sucrose were provided by Sigma. **Medium B** contains half concentration of the micro and macronutrients, 13.3g/l sucrose and 10mg/l adenine sulphate. **Medium C** consists on 13.3g/l sucrose, 0.2mg/l BA and the absence of NAA. In **medium D** the concentration of NAA and BA was 0.2mg/l, the sucrose concentration was 13,3g/l and no adenine sulphate was present. **Medium E** contains 5g/l activated charcoal, 13.3g/l sucrose, 0.2mg/l BA, no adenine sulphate and no NAA. Media C', Ca, Cb and Cd contain 10g/l sucrose and no NAA beside others modifications. **Medium C'** contains, 10mg/l adenine sulphate and 0.1mg/l BA. **Medium Ca** contains 0.1mg/l BA and no adenine sulphate. In **medium Cb** no BA and no adenine sulphate were present. **Medium Cd** contains 10mg/l adenine sulphate and no BA. Media G, Ga, Gb and Gd contain half concentration of the micro and macronutrients, 6.7g/l sucrose and no NAA beside others modifications. **Medium G** contains 5mg/l adenine sulphate and 0.05mg/l BA. **Medium Ga** contains 0.05mg/l BA and no adenine sulphate. In **Medium Gb** no BA and adenine sulphate were present. **Medium Gd** contains 5mg/l of adenine sulphate and no BA. **Medium 1/2G** consists on a quarter of micro and macronutrients, 3.3g/l sucrose, 2.5mg/l adenine sulphate and 0.025mg/l BA. Finally **medium 1/4 G** contains 1/8 of micro and macro nutrients, 1.6g/l sucrose, 1.25mg/l adenine sulphate and 0.0125mg/l BA.

### *Experimental design*

This study was conducted in glass jar of 10cm heightx10cm diameter containing 120ml of medium. Seven nodal cutting stem explants about 1cm long, were subcultured in each jar. Testing the 32 clones at the same times in different medium condition was not manage-

**TABLE 2.**

**Experimental design**
**Eight series of different media were tested in parallel with different clones.**

| Series number | Media tested | Number of different clones tested |
|---|---|---|
| 1 | A, B, C, D, E | 10 |
| 2 | A, B, C, D, E, C' | 5 |
| 3 | A, B, C', E | 10 |
| 4 | C', Ca, Cb, Cd | 16 |
| 5 | A, B, C', G, Ga, Gb, Gd | 7 |
| 6 | C', G, Ga, Gb, Gd | 7 |
| 7 | A, B, C', Ca, Cb, Cd, G, Ga, Gb, Gd | 7 |
| 8 | G, 1/2G, 1/4G | 12 |

able, therefore, clones and media were tested in 8 temporal series initiated along one year as it is shown in Table 2. Firstly, we reduced macro and micronutrients, sugar and growth regulators and we tested the effect of activated charcoal. Then few media were discarded and growth regulator combinations are tested in two basal media, C' and G. Finally a stronger reduction of macro and micronutrients, sugar and growth regulators (media 1/2G and 1/4G) were achieved. Different media and clones were used in each series, in the series 1, 2, 7 and 8, two jars for each clone and media were used; 90-100% repetition of the results were obtained. Media A, C and/or G were used as control.

*Recorded data*

First observations were performed one month after setting on the explants. For each explant the following data were recorded: a) the presence or absence of callus formation per nodal stem in each media was counted and expressed as a percentage, b) the number of shoots per stem explant in each jar, c) the size of the shoots - expressed in cm and d) the root size per explant in each jar, after considering three root categories: i- little roots mean roots from 0 to 1cm long, ii- medium roots mean roots from 1 to 5cm long, and iii- extensive roots mean roots over 5cm long.

Three months after subculturing we registered again the parameter mentioned above. In most cases, three month old shoots were too big and interspersed with each other; it was not possible, therefore, to characterize them individually and for survival culture evaluation the whole plantlets of each jar were taken into account. Culture evolution was controlled monthly, following the same visual criteria as we used for the maintenance of the collection. Subcultures were monthly checked for eight months; percentages of survival where estimated. Then the survival shoots were used for the collection maintenance.

*Statistical analysis*

Each experiment was carried out at least two times with seven stem explants per treatment. Data were subjected to analysis of variance (ANOVA) with a $P<0.05$.

## Results

*Number of shoots per explant*

The results of the nodal cutting stem explant growth in the 15 culture compositions after one month were compiled in Table 3. The average of shoots induced per explant was 1-2 in the media without BA: Cb, Cd, Gb and Gd, and lower than one in medium D with a ratio BA/NAA of one. In media E, 1/2G and 1/4G with a reduction of BA, sucrose and macronutrients, around two shoots per explant were observed. Media A, C, C', Ca, G and Ga with a proportional reduction of BA, sucrose and macronutrients, induced more than two shoots per explant. In most of media, clonal variability in shoot production was observed, e.g. clone 10SALC produced a high number of shoots with very small leaves and poor roots in all media tested. Other clones (01VILL, 04FRES, 07CELA, 08OTER, 10SALC, 40VBTO and 42CAAR) produced more than four shoots per explant. The opposite was observed with clones 02CONG, 11RESP and 36TRIO, that gave only one shoot per explant in the same period of time.

In medium C', however, most of the 32 clones had a similar behavior; the average number of shoots, their growth after one and three months, their rooting and callus formation were represented on Table 4.

**TABLE 3.**

**Growth in the 15 media after 1 month of subculture**
**Number of shoots: average number of shoots per stem. Average length of shoots. Three categories of roots were established depending on their length (< 1cm, 1-5cm, >5cm); number represent percentage.**

| Media | A | B | C | D | E | C´ | Ca | Cb | Cd | G | Ga | Gb | Gd | 1/2G | 1/4G |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of shoots | 3.1 ± 0.4 | 2.5 ± 0.4 | 4.2 ± 1.1 | 0.9 ± 0.2 | 2.5 ± 0.6 | 3.1 ± 0.4 | 4.1 ± 0.6 | 1.5 ± 0.2 | 1.2 ± 0.1 | 3.7 ± 0.3 | 3.6 ± 0.4 | 1.4 ± 0.2 | 1.3 ± 0.2 | 2.6 ± 0.5 | 2.1 ± 0.4 |
| Length of shoots (cm) | 1.5 ± 0.4 | 1 ± 0.2 | 2.2 ± 0.7 | 0.3 ± 0.1 | 1.7 ± 0.5 | 1.3 ± 0.2 | 1.5 ± 0.2 | 2.5 ± 0.3 | 1.9 ± 0.5 | 1.9 ± 0.2 | 1.2 ± 0.2 | 3.1 ± 0.3 | 3 ± 0.4 | 1.7 ± 0.4 | 1.9 ± 0.3 |
| % little roots (<1cm) | 85 | 97 | 86 | 33 | 75 | 94 | 100 | 80 | 100 | 94 | 96 | 85 | 77 | 100 | 100 |
| % medium roots (1-5cm) | 12 | 3 | 14 | 33 | 25 | 6 | 0 | 20 | 0 | 6 | 4 | 15 | 23 | 0 | 0 |
| % extensive roots ( >5cm) | 3 | 0 | 0 | 33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Shoot size*

After one month of subculture, shoots sized 1-2.5cm in all media except D (0.3cm) and Gb and Gd (3.1-3cm, Table 3). After three months shoots in media C, Cb, G, Ga, Gb and Gd reached more than 4.5 cm. Shoots grew uniformly (1.5-2cm/month) during the three first months after subculture on media C, C', Ca, G and Ga. In media Cb and Gb the growth was particularly stimulated (5.8cm). On the contrary, the culture conditions of medium D produced a few numbers of shoots with a low growth; after three months they reached less than 1cm height. In general, media without BA (Cb, Gb and Gd) induced a quicker growth during the first month, and slower afterwards. In medium E, the growth showed a similar pattern but the shoots were shorter, they reached 1.7cm after one month and 3cm after three months (Fig. 1). Medium E which contained 0.5% of activated charcoal produced chlorotic leaves indicating that the charcoal adsorbs essential nutrients for the metabolism of plants. It seems that *P. tremula* from our collection did not exude toxic chemical that could alter the maintenance of the clones during these periods of time. In media 1/2G and 1/4G, the growth was moderate but the stems were thinner and weaker. Note that beside a general behavior, clones 01VILL, 05FONT, 08OTER, 19VIDR and 25VELI had the fasted growth while clones 11RESP, 20VALC and 47SALI had the slowest growth (Table 4).

*Roots*

Initial roots were a general feature after one month of subculture in most of the media (Table 3). Medium roots were present in a lower percentage except media Ca, Cd, 1/2G and 1/4G. Except medium D (33%) and with a low percentage, medium A (3%), did not induce long roots in this short period. After three months, more than 50% of the shoot roots reached more than 5cm in all medium conditions, excepted in media Cd, 1/2G and 1/4G. After one month, medium roots were observed only with three clones (03LAST, 05FONT and 25VELI); the other clones had initial roots. After three months, 19 clones continued having initial roots whereas clones 05FONT, 13VALS, 22RIOS and 45QUIN reached extensive roots. The other clones produced medium roots (Table 4).

Media D, A and B that contain exogenous NAA induced the highest percentage of callus, 61%, 34% and 28% after one month, and 86%, 53% and 51% after 3 months, respectively (Fig. 2). Even without exogenous NAA, media C', Ca, Cb, G and Ga induced callus formation after three months, 6%, 18%, 4%, 5% and 4.7%, respectively. No callus was induced with media C, E,

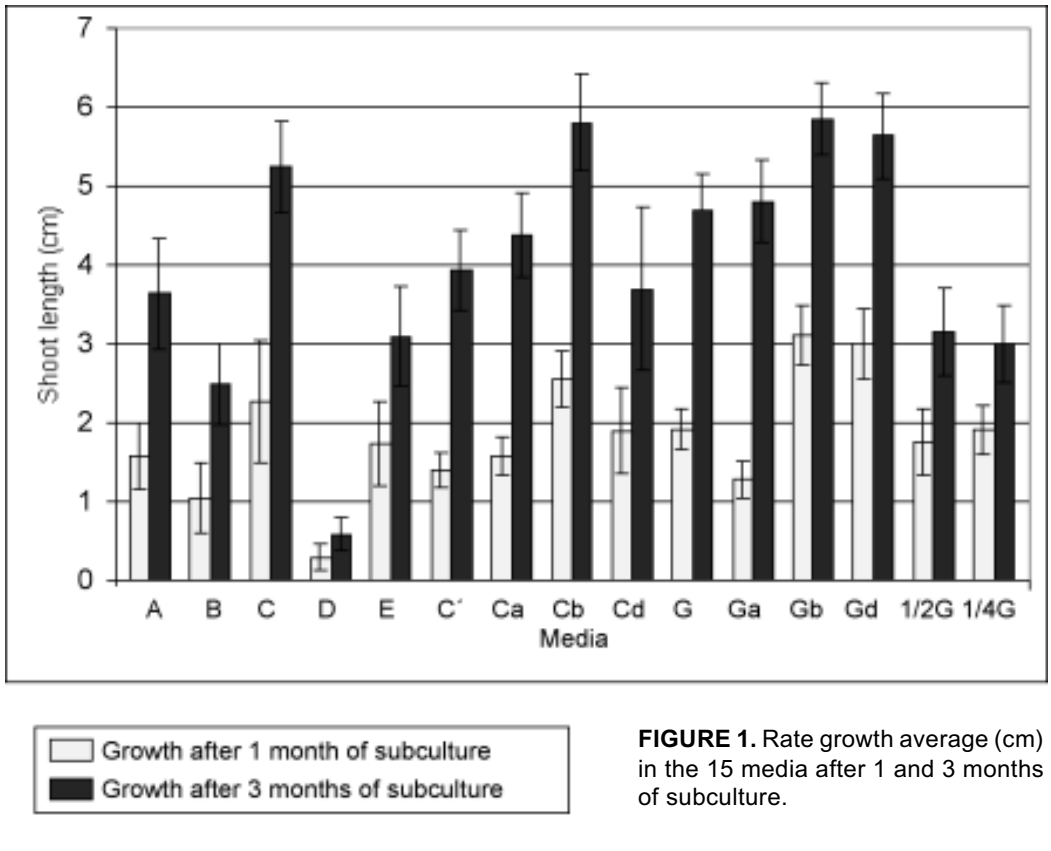


**FIGURE 1.** Rate growth average (cm) in the 15 media after 1 and 3 months of subculture.

Cd, Gb, Gd, 1/2G and 1/4G. Clones 01VILL, 07CELA, 14CAMP, 35FRES and 40VBTO produced callus after one month of subculture. Twelve clones produced callus by medium C' after three months of subculture, only in one or two explants; therefore, the percentage shown in Figure 2 only reached 6%.

*Survival*

The survival decreased more or less progressively during the eight month of the experiment. Figure 3 (A and B) shows the survival rate of shoots in the 15 media tested. The best survival rate was obtained by media

**TABLE 4.**

**Behavior of the 32 clones growth in medium C'**
**Growth characteristic for each clone: number of shoots per explant after one month of subculture, high of the shoots, length of the roots and callus inductions after one and after three months of subculture were noted.**

| CLONES NAME | Number of shoots / explant | Growth (cm) | | Presence of roots | | Presence of callus | |
|---|---|---|---|---|---|---|---|
| | | 1 month | 3 months | 1 month | 3 months | 1 month | 3 months |
| **01VILL** | 4.5 | **1.25** | **5.75** | Little | Medium | **Yes** | **Yes** |
| 02CONG | 1 | 0.5 | 3.5 | Little | Little | No | No |
| 03LAST | 3 | 2.25 | 4.25 | Medium | Medium | No | No |
| 04FRES | 4.5 | 1.5 | 4.5 | Little | Medium | No | Yes |
| **05FONT** | 2.5 | **1.5** | **6** | Medium | **Extensive** | No | No |
| 06DEHE | 2.5 | 1.75 | 3 | Little | Medium | No | No |
| **07CELA** | **5** | 1.5 | 4 | Little | Medium | **Yes** | **Yes** |
| **08OTER** | 4.5 | **1.75** | **6.25** | Little | Medium | No | No |
| **10SALC** | **7** | 2 | 4.25 | Little | Medium | No | No |
| **11RESP** | 1 | **0.5** | **0.5** | Little | Little | No | No |
| **13VALS** | 4 | 2 | 5 | Little | **Extensive** | No | No |
| **14CAMP** | 4 | 1 | 3 | Little | **Little** | **Yes** | **Yes** |
| 16MANT | 3 | 1.5 | 3 | Little | Medium | No | No |
| 17BCAS | 2 | 2.5 | 5.25 | Little | Medium | No | Yes |
| 18BCAM | 2.5 | 1.75 | 2.25 | Little | Medium | No | Yes |
| **19VIDR** | 3 | **1.5** | **7** | Little | Medium | No | No |
| **20VALC** | 2 | **0.3** | **1.5** | Little | Little | No | No |
| 21CANT | 3 | 1.7 | 3.7 | Little | Medium | No | Yes |
| **22RIOS** | 2.5 | 0.5 | 5 | Little | **Extensive** | No | No |
| 23SANF | 2.7 | 1 | 2.5 | Little | Little | No | No |
| **25VELI** | 1.5 | **1.75** | **6** | Medium | Medium | No | No |
| 27VELI | 3 | 1.25 | 4 | Little | Medium | No | Yes |
| 28CANA | 4 | 2 | 5 | Little | Medium | No | No |
| 29CANA | 2.5 | 0.5 | 2.25 | Little | Little | No | No |
| 34CANA | 4 | 1 | 5 | Little | Little | No | No |
| **35FRES** | 4 | 2 | 3.5 | Little | Medium | **Yes** | **Yes** |
| 36TRIO | 1 | 1 | 2.5 | Little | Little | No | No |
| **40VBTO** | 4.5 | 2.5 | 4.75 | Little | Medium | **Yes** | **Yes** |
| 42CAAR | 6 | 1 | 4.25 | Little | Medium | No | Yes |
| **45QUIN** | 2.5 | 1.25 | 2.5 | Little | **Extensive** | No | No |
| **47SALI** | 2.5 | **1.3** | **1.7** | Little | Little | No | Yes |
| 50SMTI | 3 | 0.8 | 5 | Little | Medium | No | No |

Ca, Cb and Gb, 70%, 65% and 62%, respectively. Even more, subculture into media B, C, C', G and Ga presented higher percentage of green shoots than medium A, with around 50% of survival after 8 months of subculture. The lower percentage of survival was obtained with media D, E, A and Cd (9%, 22%, 31%, and 39%, respectively). In media E, 1/2G and 1/4G, the shoots were weak and chlorotic probably due to a high reduction of nutrients and the presence of activated charcoal in medium E. Clones (06DEHE, 07CELA, 08OTER, 34CANA and 35FRES) maintained green leaves and stems beyond eight months.

## Discussion

We studied 15 different chemical compositions of *in vitro* culture media that allowed the conservation of healthy plants with vigorous stems, green leaves, developed roots and slow growth avoiding callus formation, during a period of time superior of three months without subculture.

Medium A is optimal to produce buds and subsequent shoots in a number high enough for micropropagation, as previously reported (Ahuja, 1983; 1987). It is remarkable that media C and Ga yielded more shoots than A with our 32 clones (4.2 and 3.6, respectively) after one month of subculture. A high number of shoots was also obtained with C', Ca and G media; these media contain less concentration of adenine sulphate and BA than medium A. Our results indicate that exogenous NAA is not required and 0.05mg/l BA is enough to induce shoot formations.

The proportion among salts, sucrose and growth regulator compounds were compared in different media. As it was expected, the absence of BA reduced the number of shoots per explants (media Cb, Cd, Gb and Gd) (Douglas, 1985). Adenine sulphate reduced the growth in Cd but not so much in Gd, probably due to interaction among salts, sucrose and adenine sulphate concentrations. The growth in media G, Gb and Gd was highest than in media C', Cb and Cd. Medium C' gave a slower growth than the 7 other media and medium C. This characteristic is needed for preservation.

In medium C' root formations were obtained for 23 clones during the three first months of subculture without exogenous NAA whereas 9 clones still showed initial roots. It was assumed that *P. tremula* has trouble for rooting; however, clones 05FONT, 13VALS, 22RIOS and 45QUIN reached high roots in three months.

Calluses are mainly formed by dedifferentiated cells, a mechanism that could be induced by different ratios of NAA/BA as it is observed in the three following examples. The proportion NAA/BA equal to 1 used

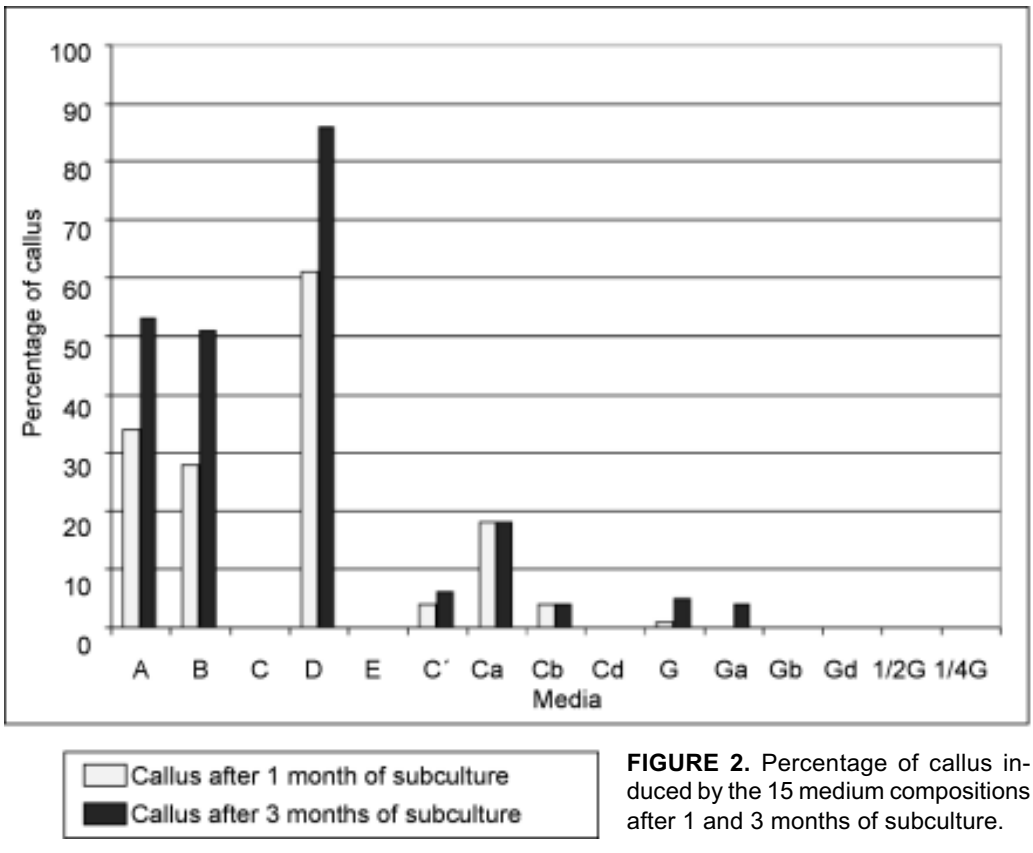


**FIGURE 2.** Percentage of callus induced by the 15 medium compositions after 1 and 3 months of subculture.

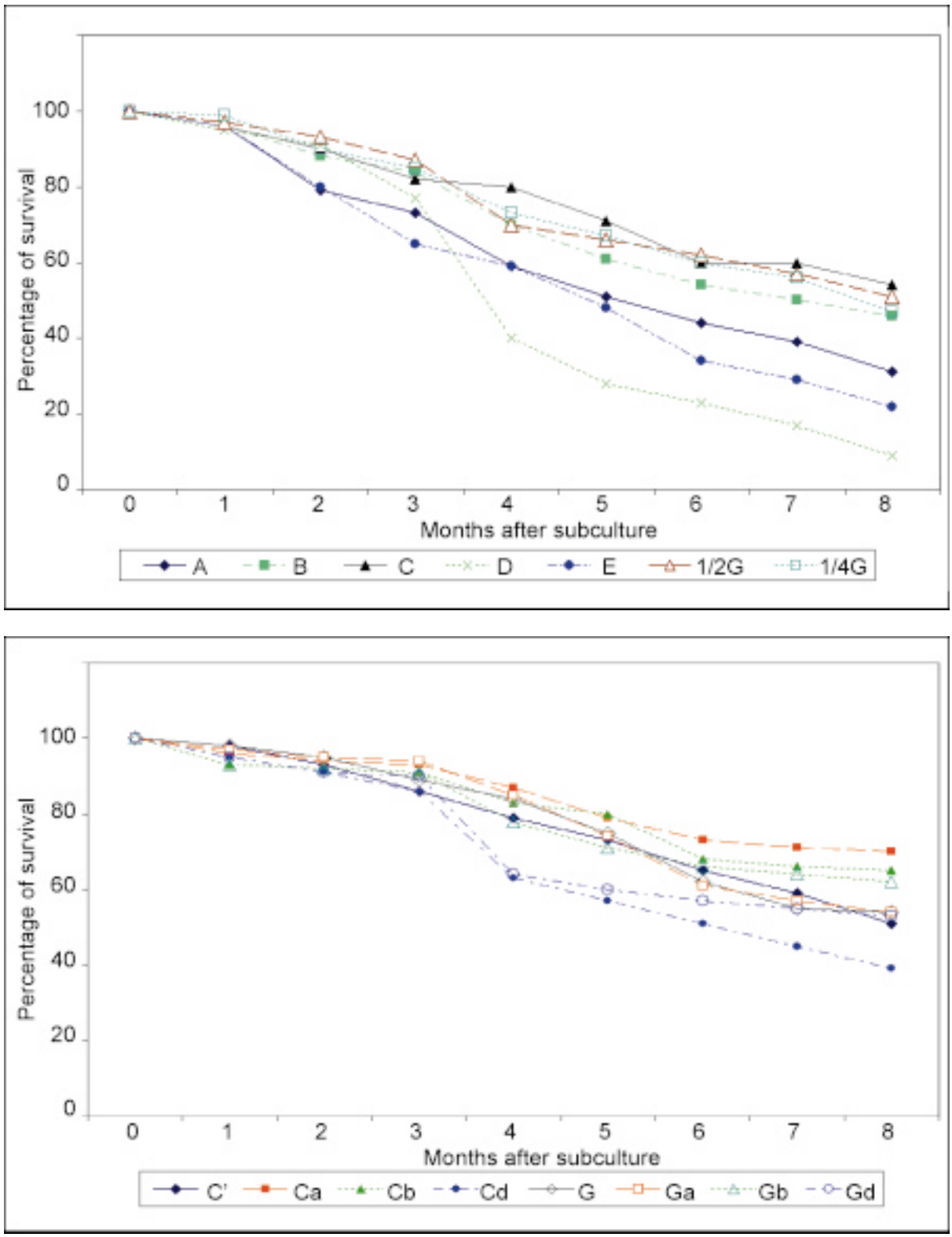


**FIGURE 3.** Percentage of survival.
Evolution of green shoots during 8 months, in the 15 media tested. Each point represented the mean value of the 32 clones. A) Survival in the following 7 media: A, B, C, D, E, 1/2G and 1/4G with decreasing macronutrients and sucrose concentrations and modification of growth factors ratios. B) Comparison of the growth factors (presence and/or absence of adenine sulphate and/or BA) effects on the survival under the composition media C' and G.

in medium D of this study, or the proportion NAA/BA of 0.3 described by Ahuja (1983) or the proportion of 2.2 described by Antonetti and Pinon (1993). After dedifferentiation, a new organogenesis allows new shoots and plant formation with, in some cases, somaclonal variation; therefore, the genetic fidelity is endanger. High occurrence of callus formation led to discard media A, B and D, particularly for clones 01VILL, 07CELA, 14CAMP, 35FRES and 40VBTO that were prone to form callus. The media C', Ca, Cb, G and Ga induced a lower callus percentage; the risk of genetic alteration was reduced.

After 8 months of subculture, among the 15 media used, the best survival was obtained with medium Ca, double compared with that obtained with A. Only media E and D resulted in worse survival than A. Media that gave higher survival but produced weak reclining stem like 1/2G, 1/4G, Cb, Gb and Gd, were not appropriated for successive subculture; those media were discarded for *P. tremula* preservation. These results and the inventories made, showed that clones 06DEHE and 07CELA had a longer survival than the other clones, indicating other variables beyond genotype might mediate differential behavior among clones.

In conclusion, as our aims were to obtain a medium for conservation purposes, media A, B, C', D, E, Ca, Cb, Cd, Gb, Gd, 1/2G and 1/4G were discarded because they induced callus, produced thin and weak stems, fast growth, reduced number of shoots and/or led to a low survival rate at the end of the experiment. In contrast, media C, G and Ga might be good options, medium Ga is the cheapest one. Medium Ca is the best option for maintenance of clones that have a poor ability to form callus since the highest survival was obtained in it. Subculture into the 15 media allowed a further characterization of our 32 clones in *in vitro* conditions. Their differential behavior suggests that they probably have a high degree of genotype biodiversity; this topic requires further analysis.

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