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The establishment of an *in vitro* culture protocol for *Calamagrostis intermedia* (J. Presl) Steud. starting from meristems

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Abstract

The Andean Paramo is an important component of biodiversity, affected by the species inhabiting it and by the human activities. For these reasons the biotechnological technique represents an important tool for helping the conservation of this ecosystem, one of the alternatives being represented by the in vitro culture. This research aims to establish a protocol for in vitro micro propagation of Paramostraw (Calamagrostis intermedia) represented by the disinfection method and choose of the introduction, multiplication and rooting media, for short term conservative issues. For the disinfection phase were tested three concentrations of sodium hypochlorite(0.5%, 1%, 1.5%)(v/v) with different immersion time(10 to 15 minutes).The introduction media was represented by seven MS media with different concentration of vitamins: 0%, 15%, 30%, 45%, 60%, 85% and 100% (v/v). In multiplication and rootingphase was tested the influence of phytohormones such BAP (0.15 and 0.2 mg/L), BRA (2 and 3 mg/L) and KIN (0.05 and 0.1 mg/L). The best treatment for disinfection phase was represented by sodium hypochlorite 1% for 10 minutes. The important results for the micro propagation of this species consisted in using a concentration of vitamins of 30% (v/v) in MS medium during the introduction phase and 3 mg/L of BRA in the phase of multiplication and rooting.

Keywords: *Calamagrostis intermedia*(J. Presl) Steud., Ecuador, conservation, ParamoAndino.

1. Introduction

Paramo straw, (*Calamagrostis intermedia*), is a monocot plant characterized by dense clumps. The morphology of the plant shows that the leaves have a cylindrical shape due to the edges that are folded and the inflorescence is composed. The localization of this species is in the paramostarting from Colombia to Argentina. (Ulloa *et al*., 2004)

The Andean Paramo is an ecosystem located between the Sierra Nevada of Santa Marta in Colombia, the Cordillera de Mérida in Venezuela until Huancabamba depression in Peru, constituting an important component of biodiversity in each crossing country. This ecosystem called páramo in Ecuador, known as grassland, has an especial diversity due to three main factors: the equatorial situation, the presence of the Andes and the presence of other smaller saws (Mena & Hofstede, 2006). In the case of Ecuador the paramo covers about 1.25 million ha (6% of the national territory) which mean that Ecuador is the country with more over moorland in relation to the total area of the country (Medina & Mena, 2001).

The mobilization of diverse populations to the area of the paramo has made an impact on local species, this occurred due to the displacement that was caused by the settlers in both the Sierra and the Coast in Ecuador, causing the mobilization of native people to high lands which slowly start causing that the natural wilderness habitat has been reduced and also the number of individuals per species in the area (Mena *et al*., 2008).

One of the most important ecosystem impacts comes from livestock, as this not only consume the flora of the area, but the weight of the animals and the hulls have the same results in the alteration of the soil. The burning of vegetation of the paramo with the purpose of having young plants, for the use of feeding livestock species also decrease the species that are originally found in this area (Mena & Hofstede, 2006).

All these problems create the need to generate alternatives for the recovery and conservation of the species that are affected. One alternative is the *in vitro* culture, which allows the propagation of plants more quickly allowing in this way to recover the population of different species (Paunescu A., 2009; Manole A.*et al.*, 2015).Therefore, this research aims to establish a methodology for the introduction and spread of straw (*Calamgrostis intermedia*) by the use of *in vitro* culture techniques.

2. Materials and Methods

Selection, collection and transport of plant material

Paramo straw samples (*Calamagrostis intermedia*) were obtained in the province of Pichincha, location: 9962415.56S 810221.81E and at a height of 3967 meters (via Papallacta); Once the plant were geographically located, the next step consisted in picking the youngest plants with green leaves and a height of 50centimeters to 70 centimeters, free of pests (fungi, bacteria, nematodes). The plant materials used in the research are the meristems of the straw.

Disinfection of explants (meristems)

For the disinfection phasethe explants were rinsed in running water for 12 hours, washing next in detergent (3% w/v) for 15 minutes, iodine solution (50% v/v) 5 minutes, fungicide (1% w/v) 15 minutes, then washed in sodium hypochlorite solutions (these three are going to be tested in this phase): 0.5% (v/v), 1% (v/v) and 1.5% (v/v) at different immersion times 10 to 15 minutes, the last washing being performed on tetracycline (1 mg/ml) for 10 minutes. All washes were performed in immersion with agitation of 120 rpm. (Fig. 1 and Fig. 2). The data was taken at 30 days after the seeding.

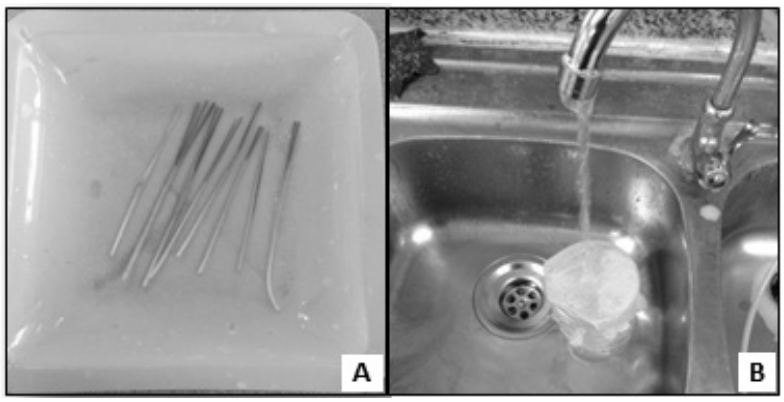

Fig 1.(A) Meristems. **(B)** Explants in running water for twelve hours.

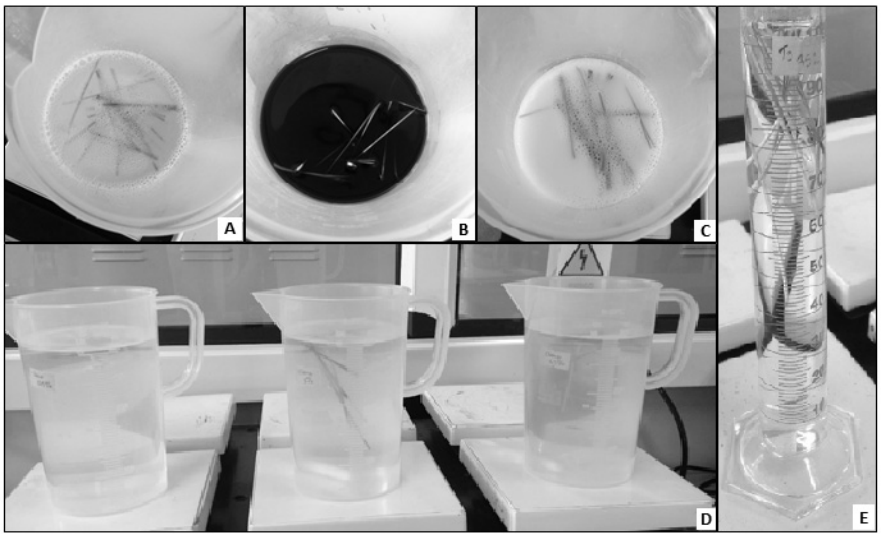

Fig 2.(A) Explants in detergent solution, **(B)** Explants in iodine solution, **(C)** Explants in fungicide solution, **(D)** Explants in in sodium hypochlorite and **(E)** Explants in tetracycline solution.

Introduction media

The culture media that was used for this part of the investigation was Murashige& Skoog (MS) but varying the concentration of the stock of vitamins, the different concentrations being specified in Table 1, BactoAgarof 8gr/L, sucrose 3 % (w/v) and a pH of 5.7 to 5.8. The data was taken 30 days after the seeding.

Table 1. Treatments that were tested in the phase of introduction of the explants (meristems) of Paramo straw.

Treatment	Vitamins concentration (% v/v)
V1	0
V2	15
V3	30
V4	45
V5	60
V6	85
V7	100

Media for the inducción of shoots and roots

The culture medium used is composed of the Murashige & Skoog (MS) salts, vitamins (stock of vitamins that belongs to MS medium) the best concentration obtained in the previous phase, BactoAgar in a concentration of 8gr/L, sucrose 3% (w/v) and a pH of 5.7 to 5.8. This media will be supplement with phytoregulators: BAP, KIN and BRA at concentrations specified in Table 2, in the case of shoot induction and number of the same, the data was taken at 15, 30 and 45 days after seeding; and rooting was measured at 30, 60 and 75 days after planting.

Table 2. Treatments tested in the phase of induction of shoots and roots in Paramo Straw'meristem.

Treatment	BAP (mg/l)	BRA (mg/l)	KIN (mg/l)
T1	0,15	-	-
T2	0,2	-	-
T3	-	2	-
T4	-	3	-
T5	-	-	0,05
T6	-	-	0,1

Experimental design

The variables measured on each phases of the investigation were represented by the contamination and oxidation during the disinfection process, viability in the phase of media introduction, absence or presence of shoots and roots, the number of shoots and roots. The treatments tested in this research were evaluated by statistical analysis and exploratory data graph, was assessed whether the data collected following a parametric distribution or not. For this inferential analysis was used the analysis of variance (ANOVA), with its assumptions: graphic of QQ plot and the Shapiro-Wilks modified; if using these tests it was not found that the data do not follow a parametric distribution, a Kruskal-Wallis was performed. To perform statistical tests were used as statistical software InfoStat and RapidMiner.

3. Results

Disinfection of explants (meristems)

In this phase contamination data (Fig 3) and oxidation of the explants was evaluated, in the case of oxidation this was not present in any of the treatments.

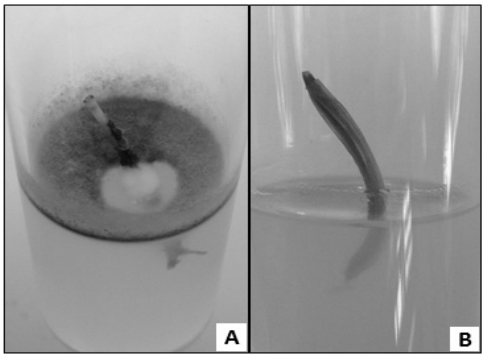

Fig 3. (A) Explant with contamination and **(B)** Explant with no contamination.

The concentration of 1% and an immersion time of 10 minutes in sodium hypochlorite (Treatment D2) allowed a more effective disinfection of explants and had a higher viability of the meristem then the other disinfection treatments. At a lower concentration of sodium hypochlorite, the presence of contamination was higher, whereas if the chlorine concentration was increased in time and concentration it could be detected the presence of some necrosis in the meristems (Table 3).

Although treatment D3 (1.5% NaClO concentration and a time of 10 min) showed the same results as the treatment D2 for contamination, in the treatment D3 could be seen the presence of necrosis in some explants, thus affecting the level of viability of meristems using this treatment.

Table 3. Contamination contingency.

		Concentración de cloro		
		0,5 %	**1 %**	**1,5 %**
Tiempo de inmersión	**10 min**	9	5	5
	15 min	18	9	19

Introduction media

In this phase the viability of the explant was evaluated in MS media in which was varied the concentration of vitamins. (Fig 4)

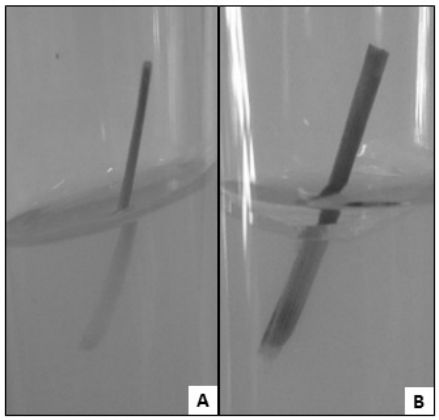

Fig 4. (A) Viable meristem of Paramo straw. **(B)** Not viable meristem of Paramo straw.

The modified media with a percentage of 30% vitamins (V3) allowed a higher percentage of viability in explants (meristem) of Paramo straw. If the vitamins were present in a lower concentration, V1 and V2 treatments, there was a high percentage of viability, 66.67% and 60.00% respectively. A higher concentration of vitamins represented by the treatments V4, V5, V6 and V7, indicated that the percentage of viability is lower 33.33%; 46.67%; 46.67% and 40.00% respectively.

Table 4 present the number of explants (meristem of Paramo straw) that are viable in each of the treatments and the viability percentage held by each of the treatments after 30 days from sowing. The best treatment identified is V3 with 93.33% viability and the other treatments have a lower percentage of viability, the lowest treatment being represented by V4 with a percentage viability of 33.33%.

Tabla4. Viability of meristems of Paramo Straw in each treatment, with the variation of the concentration of vitamins in MS media

Treatment	**Concentration of vitamins (%)**	**Viable explants**	**Porcentage of viability (%)**
V1	0	10	66,67
V2	15	9	60,00
V3	30	14	93,33
V4	45	5	33,33
V5	60	7	46,67
V6	85	7	46,67
V7	100	6	40,00

Media for shoots induction and multiplication.

At this phase were tested the presence and absence of shoots and the number of shoots present in each explant seeded (Fig. 5).

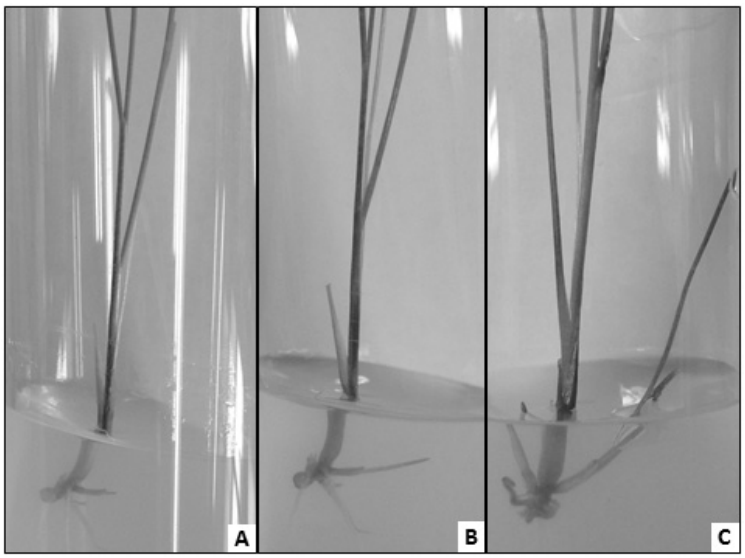

Fig 5.(A) Shoot at 15 days, **(B)** Shoot at 30 days and **(C)** Shoots at 45 days

The use of brassinolides at a concentration of 3 mg/L (Treatment T4) in the culture media set in the second phase, allowed a greater presence of buds explants of Paramostraw and the use of 2 mg/L (Treatment T3) brassinolides involved the presence of shoots. In the other treatments used (Treatments T1, T2, T5 and T6) the presence of outbreaks after the evaluation time was not present.

The treatment T4 (Brasinolidas 3mg/L) was showing the highest presence of outbreaks after completing 45 days (last day of data collection), the next treatment in number of outbreaks being represented by T3 (Brasinolidas 2mg/L), although the number of outbreaks is lower than treatment T4 after 45 days of planting. The other data recorded indicate the lack of the outbreak after the 45 days cultivation in the treatments T1, T2, T5 and T6.

The total number of shoots per treatment tested can be seen in Table 5,the best treatment being represented by T4 with 12 shoots in total of all 15 seeded explants combined, followed by T3 treatment with 3 outbreaks, while the other treatments (T1, T2, T5 and T6) showed no outbreak.

Table 5. Number of outbreaks in each treatment tested at the period of 15, 30 and 45 days.

Treatments	Shoots at 15 days	Shoots at 30 days	Shoots at 45 days
T1	0	0	0
T2	0	0	0
T3	1	3	3
T4	4	6	12
T5	0	0	0
T6	0	0	0

Media forshootrooting

At this phase were evaluated the presence and absence of root and the number of roots per shoots (Fig 6), being observed that the addition of brassinolides at a concentration of 3 mg/L (T4) to the media established in phase II, allowed a greater regeneration of roots in meristems seeded of Paramo straw. It was also observed that placing brassinolides in concentration of 2 mg/L (T3) to the media induce the presence of roots in smaller amount. In the other treatments used (T1, T2, T5 and T6) was not seen the presence of root once the evaluation time has finished.

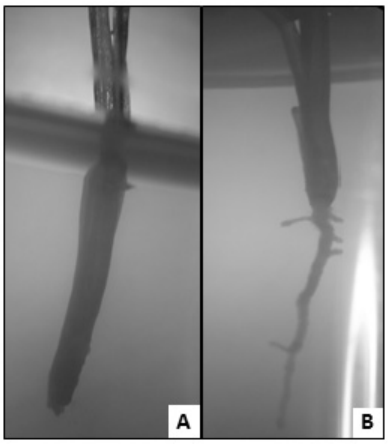

Fig 6. (A) Shoot without root formation and**(B)** Shoot with root formation.

The total number of roots for each treatment tested is represented in Table 6. The best treatment was achieved by T4, that induced 14 roots in total of all 15 explants that were seeded, followed by treatment T3 that present 7 roots. In the other treatments (T1, T2, T5 and T6) was not observed the root formation.

Tabla 6. Number of roots in each treatment after 30, 60 y 75 days since the explants were seeded.

Treatment	Roots at 30 days	Roots at 60 days	Roots at 75 days
T1	0	0	0
T2	0	0	0
T3	0	6	7
T4	0	10	14
T5	0	0	0
T6	0	0	0

4. Discussion

Many studies in the case of monocots investigated by Norciniet al. (2003) and Calderón-Arias et al. (2011) proved first the presence of callus and then of shoots (indirect organogenesis), but in this study from previous tests was showed the lack of the callus regeneration from leaves, meristems or inflorescences, so we proceeded to conduct the research for direct organogenesis with the use of meristems as explants. One of the facilities of this plant is that it grows sprouts with the name of stolons (Sklenář*et al*., 2005) which allows multiplying the plant later.

For the explant selection, it was decided to use the meristem of the plant, this because of the characteristics that have the explants in general as stipulated by Khachatourianset al. (2005) for the selection of the explant, so it is required a high rate of cell division and a high plasticity of the presenting cells, in the case of monocots the explants fitting to this two requirements being the immature organs and meristems. For this reason, it was decided to collect the meristems of young plants and new growth shoots.

For the disinfection phase it was used the protocol proposed by Calderón-Arias et al. (2011) with modifications, the best results being obtained by the use of sodium hypochlorite 1% (v/v) for 10 minutes with a high percentage of explants no contaminated;

At the base of the induction of outbreaks phase was the study made by Martinez et al. (2012) in which BAP and KIN were used with good results for the induction and development of shoots, but in this investigation meristems of Paramo straw had no positive effect using these phytohormones, showing a lack of shoot regeneration in the meristemsseeded.

The use of brassinosteroides it was based on the study of Capote et al. (2009) which indicates that brassinosteroides are a group of steroids that serve for a wide range of plant processes, this being confirmed in our research by the shoots and roots regeneration with the addition of 3 mg/L of BRA to the media and also 2 mg/L of BRA, although the second one causes more effect on the shoots and roots elongation than on induction.

5. Conclusions

It is important to standardize a protocol for *in vitro* culture of Paramostraw (*Calamagrostis intermedia*) starting from explants represented by the meristems. The best treatment in the disinfection protocol was represented by 1% (v/v) hypochlorite sodium with an immersion time of 10 minutes and the treatment for the introduction media with the best viability was MS with 30% (v/v) of vitamins; 3 mg/L of Brassinolides was the concentration used for the induction of shoots and roots with the best results for the conservation issues of this important species of paramo.

6. Acknowledgments

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