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Deliverable: D17251 - Report on In Vitro propagation from the Bh16 Collection of Urochloa humidicola from seed.

Report *on In Vitro* propagation from the Bh16 Collection of *Urochloa humidicola* from seed.

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ABSTRACT

The procedure of disinfection and in vitro sowing of a set of materials corresponding to the BH16 group of Urochloa humidicola was carried out at CIAT headquarters, with this it was possible to establish In Vitro plants without high percentages of contamination by fungi or bacteria, in addition to finding that the use of sulfuric acid for scarification procedures before sowing generates germination results similar to those that would be obtained in silica sand. This being a significant and pioneering advance in the standardization of In Vitro propagation methods for U. humidicola.

INTRODUCTION

The term *In Vitro* culture in plants means to cultivate isolated parts of the plant, inside a glass jar in an artificial environment, in this way, the growth and development of various plant materials is promoted in containers that keep it isolated from the outside environment and allows the management of its conditions being controlled and aseptic (absence of germs) (Borges et al, 2009) (Zurita et al, 2014).The propagation of these new plants is favored due to the rapid growth of the plant material thanks to the components of the medium where they are grown and the moisture it contains (Murashige and Skoog, 1962).

Thus, the establishment of *In Vitro* cultures by sowing seeds offers important advantages for propagation: 1) it quickly provides seedlings that serve as a source of explants to carry out micropropagation; 2) it is a way to conserve seedlings with natural genetic variability, and 3) it is a method that allows the germination of seeds that do not naturally or is very difficult to do under normal conditions (Fay, 1992; Pierik, 1993). Additionally, in vitro germination has advantages compared to that produced in natural conditions, since it can solve cases of total inhibition of germination, increase the germination rate, reduce the time and homogenize germination (López & González, 1996); However, one of the greatest difficulties that the successful establishment of in vitro culture presents is the presence of contaminating microorganisms, therefore, it is necessary to apply protocols that lead to minimizing or eliminating said microorganisms without affecting the viability of the cultures (García et al, 2015).

In the case of *Uroclhoa*, there are few effective protocols to control pathogens that are considered quarantine and prevent their easy distribution (Casaya, T. 2004.), although methodologies are being

developed for the disinfection of seeds and vegetative material in In Vitro crops that can guarantee the international shipment of this type of materials in Colombia, there is still no effective route for it (Aranzales, E., et al., 2016), for which, the Bh16 group was taken, an improved group of *Urochloa humidicola* (generation of 2016), which is about to enter its commercialization stage by Grupo Papalotla, a Mexican company dedicated to this work (Hare et al., 2007), for which its *In Vitro* propagation is necessary in correct aseptic conditions, as an alternative for sending the Hybrid group, this being the objective of this research.

MATERIALS AND METHODS

Two *In Vitro* sowings were carried out at CIAT headquarters, located at Km 17, of the recta Cali - Palmira, in Colombia, using CIAT's tissue culture laboratory facilities. The seed used was stored in the Tropical Forages Genetics Laboratory in a cold room, which maintains a temperature of 8 °c and a RH of 50%, said seed corresponds to the hybrid group Bh16, of which the quality, in terms of percentage of Germination in silica sand (ISTA, 1999) (Natera, JRM, Moreno, MJ, & amp; Moya, JF, 2009), Viability (Ruiz, MA 2009) and the storage time is 3 years, variables that are reported in Table 1.

Seed selection

The seeds were selected based on the availability present in group Bh16, in this case, for sowing 1, 23 hybrids were selected as is shown in table1, and for sowing 2, 7 hybrids were selected, to reinforce the plants that have them as an option for *In Vitro* delivery, this information can be viewed in Table 2.

Escarification with H_2SO_4

This chemical scarification with sulfuric acid (García, J. and Cícero, SM 1992), was performed in the sowing 2, to check if the germination of the seeds in this way was faster, this process was carried out by placing the seeds in a beaker for a time of 15 minutes, the seeds were constantly stirred, then the acid was neutralized by washing with abundant water and finally they were dried on a tray with paper at room temperature, as shown in figure 1

Seed disinfection

To disinfect the seeds that would be sown *In Vitro* later, for sowing 1, caryopses of *Urochloa humidicola* were used, from which the caryopsis had been manually removed, leaving only the caryopsis of these seeds; While for sowing 2, scarification was carried out with H_2SO_4 which is explained in the previous item. These seeds were disinfected by using broad – spectrum fungicides, Ethanol, sodium hypochlorite and liquid soap (Flores, Á. B., Personal communication), this procedure was carried out on July 23, 2020 for the sowing 1 and August 14, 2020 for sowing 2.

Figure 1. Scarification of *Urochloa* seed with 96% H_2SO_4, a) Sulfuric Acid concentration of 96%, b) Agitation of seeds for 15 min, c) Neutralization of the acid with water, d) Seeds dried on paper at room temperature.

In Vitro sows

Once the seed had been disinfected, In Vitro sowings were carried out, in a 4E medium previously prepared with growth regulators (Sucrose Compound (2%), thiamine (2 ppm), inocitol (100 ppm), BAP (Benzyl Amino Purine 0.04 ppm), GA3 (Gibberec Acid 0.05 ppm), ANA (Naphthalene Acid 0.02 ppm), Medium Agar 4.6 g / l and with a pH between 5.7 and 5.8) (Muñoz Quijano, IF, & Reyes Sandino, HJ, 2006.), the medium was placed in test tubes No. 22 and / or Falcón tubes of 50 ml for sowing 1 and test tubes No. 12 for sowing 2 (See figure 2), these were carried out in a laminar flow chamber, using 3 forceps for dissection during seed manipulation, a lighter to flame the forceps (which were alternated after being flamed so as not to manipulate seed with the newly flamed forceps) , aluminum foil for the case of test tubes No. 22 (For the falcon tubes, the plastic cap of these was used) and Vinipel tape to seal the tubes. After sowing was finished, everything was done under aseptic conditions and the instruments were sterilized and autoclaved, this procedure was carried out on July 23, 2020 for sowing 1 and on August 14, 2020 for sowing 2.

Germination and contamination assesments.

After carrying out the *In Vitro* sowings, these were placed inside an incubator, at 28 ° C and a 12 h photoperiod of white light, 4 days after sowing an evaluation was carried out to determine the percentage of contamination by fungi and bacteria, as observed in figure 2, and this data was reported in tables 1 and 2, germination for its part is constantly being reviewed and tables 1 and 2 are recorded in the same way.

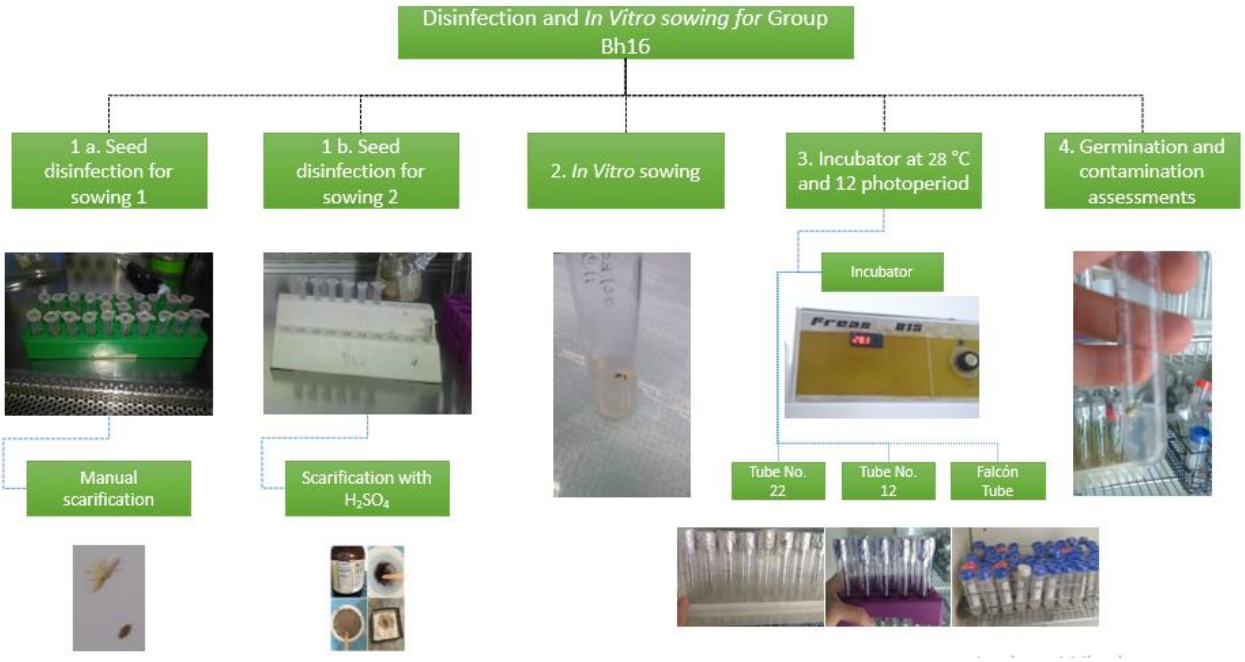

Figure 2. Disinfection and *In Vitro* Sowing Group Bh16, 1a) Show the 23 vials of 1 mL with seed scarified manually, 1b) Show the 7 vials of 1 mL with seed scarified with H_2SO_4 b) Show the 7 vials of 1 mL with seed scarified with H_2SO_4, 2) *In vitro* sowing of seed, 3) Falcón tubes and test tubes No. 22 and No. 12 Stored in an incubator at 28 °C and 12 h photoperiod, 4) Check for contamination and germination.

RESULTS AND DISCUSSION

Based on the requirements of the experiment, the following tables were constructed, in which the values of the quality variables of the seeds selected for the *In Vitro* sowings are reported, as well as the data that have been obtained so far for the variables In Vitro, including the mean and standard deviation for all variables.

Table 1. Variables evaluated for the hybrid group Bh16, before and after sowing *In Vitro* No. 1.

Group	Hybrid	Quality variables		*In Vitro* variables	
		Viability (%)	Germination in sand (%)	Germination *In Vitro* (%)	Contamination (%)
1	Bh16/181	90	40	0	0
2	Bh16/4243	95	90	0	0
3	Bh16/550	65	65	0	0
4	Bh16/2542	35	20	0	0
5	Bh16/2185	75	55	0	0
6	Bh16/1756	80	75	0	0
7	Bh16/2567	0	0	0	0
8	Bh16/3112	40	30	0	0
9	Bh16/4067	80	55	0	0
10	Bh16/562	75	50	0	0
11	Bh16/4956	40	25	0	0
12	Bh16/564	65	45	0	0
13	Bh16/1622	90	70	10	0
14	Bh16/4059	60	45	0	0
15	Bh16/503	70	40	10	0
16	Bh16/2941	85	50	0	0
17	Bh16/4970	65	55	0	0
18	Bh16/1767	60	40	0	0
19	Bh16/1763	80	60	10	0
20	Bh16/724	95	85	0	0
21	Bh16/3916	45	30	0	0
22	Bh16/3249	50	10	0	0
23	Bh16/1351	50	30	10	0
Average		*64,8*	*46,3*	*1,7*	*0,0*
Estandard dev.		*23,0*	*22,5*	*3,9*	*0,0*

** the data correspond to the last evaluation carried out on August 21, 2020.

Table 2. Variables evaluated for the hybrid group Bh16, before and after sowing *In Vitro* No. 2.

Group	Hybrid	Quality variables		*In Vitro* variables	
		Viability (%)	Germination in sand (%)	Germination *In Vitro* (%)	Contamination (%)
1	Bh16/2185	75	55	20	0
2	Bh16/2567	0	0	10	0
3	Bh16/4956	40	25	30	0
4	Bh16/564	65	45	60	0
5	Bh16/1622	90	70	0	0
6	Bh16/503	70	40	10	0
7	Bh16/3916	45	30	10	0
Average		*55,0*	*37,9*	*20,0*	*0,0*
Estandard dev.		*27,5*	*20,8*	*18,5*	*0,0*

** the data correspond to the last evaluation carried out on August 21, 2020.

In Vitro sowing No.1

When observing Table 1, we note that the procedure applied for seed disinfection is quite effective, since the average contamination percentage for the entire group is 0%, however, the germination that the

seeds have had has not been at all favorable, since it presents an average value of 1.7% and standard deviation 3.9 at 30 days after sowing, which does not resemble the behavior of the group in the viability evaluations (average 55.0% and standard deviation 27.5) and germination in silica sand (average 37.9% and standard deviation 20.8), this could be explained by the fact that the seeds were not subjected to scarification with sulfuric acid; which, in the case of *Urochloa*, has been shown to improve seed germination (Hernández Flores, E., et al., 2016), this was observed from the time of sowing on July 23, which is why It was decided to perform the sowing 2, with the groups that were not selected for the first botanical shipment of seed from Colombia to Mexico. The images of the state of the *In Vitro* plants can be viewed in the annexes.

In Vitro sowing No. 2

As already explained, this sowing was carried out adding scarification with H_2SO_4 hoping to improve the germination percentage of the seeds *In Vitro* (Hernández Flores, E., et al., 2016), when observing table 2, we note that the results that have been obtained are much more favorable with respect to sowing 1, having an average germination value of 20% and standard deviation 18.5 just 8 days after sowing, this is more similar to the germination values obtained in the germination in silica sand, which were 37.9% for those 7 groups and a standard deviation of 20.8, not to mention that it remains to continue monitoring the germinative behavior of the seeds. Images of the status of the *In Vitro* plants can be viewed in the annexes

Regarding the percentage of contamination, we note that as with sowing 1, this is 0%, which reiterates the effectiveness of the disinfection procedure recommended by the Papalotla Group, additionally it should be noted that *In Vitro* sowing is managed to have 10% germination for the Bh16 / 2567 genotype, which had presented percentages of viability and germination of 0%, which guarantees us material to replicate this genotype in the future in vitro.

CONCLUSIONS

If the possibility of carrying out the *In Vitro* shipment of the hybrid group Bh16 is taken into account (since so far this is a backup plan for the shipment of botanical seed), it is possible to guarantee the availability of all the hybrids in conditions of optimal asepsis.

BIBLIOGRAPHY

Aranzales, E., Medina, D. P., Ciprian, A., Vélez, M., & Santos, L. G. 2016. Desarrollo de metodologías in vitro para la conservación y distribución de germoplasma de Brachiaria spp.

Borges, M. Estrada, E. Pérez, I. Meneses, S. 2009. Uso de distintos tratamientos de desinfección en el cultivo in vitro de Dioscorea alata L. clon caraqueño. Revista Colombiana de Biotecnología, 11 (2): 127-35.

Casaya, T. 2004. Establecimiento del protocolo de multiplicación in vitro para pasto Toledo (Brachiaria brizantha CIAT 26110): Fase I. Trabajo de graduación. Guácimo, C R. Universidad EARTH. 46p.

Fay, M.F., 1992. Conservation of rare and endangered plants using *in vitro* methods. In vitro cellular & developmental biology - plant*,* 28: 1-4.

García, D. Mesa, N. Ocampo, M. 2015. Estandarización del protocolo de desinfección para la micropropagación de Aspidosperma polyneuron. Revista Colombiana de Biotecnología, 17 (2): 76-84

Hare, M. D., Tatsapong, P., & Saipraset, K. 2007. Seed production of two *brachiaria* hybrid cultivars in north-east Thailand. 1. Method and time of planting. *Tropical Grasslands*, *41*(1), 26.

Hernández Flores, E., Quero Carrillo, A. R., Joaquín Torres, B. M., Hernández Garay, A., & Hernández Guzmán, F. J. 2016. Métodos de escarificación y germinación en Brachiaria brizantha cv. Insurgente. *Revista mexicana de ciencias agrícolas*, *7*(1), 173-184.

Ista (International Seed Testing Association). 1999. International rules for seed testing, 1999. Seed Science and Technology 27, Supplement.

López-Encina, C., e I. González-Padilla, 1996. "A propósito de semillas". Encuentros en la Biología*,* 33: 3.

Muñoz Quijano, I. F., & Reyes Sandino, H. J. 2006. *Efecto de reguladores de crecimiento, L-Cisteína y ácido ascórbico en el cultivo in vitro de mora de castilla (Rubus glaucus benth)* (Doctoral dissertation, Universidad Nacional Agraria, UNA).

Murashige, T., Skoog, F. 1996. A revised medium for rapid growth and bioassays with Tobacco tissue cultures. Physiologia Plantarum. Vol. 15.

Natera, J. R. M., Moreno, M. J., & Moya, J. F. 2009. Efecto de diferentes combinaciones de sustratos (arena, suelo y/o bagazo de caña de azúcar) sobre la germinación de semillas y altura de plantas de guayaba (*Psidium guajava L*.). Revista Científica UDO Agrícola, 9(1), 121-125.

Pierik, R.L.M. 1993. "Micropropagation: Technology and Opportunities". Plant biotechnology commercial prospects and problems. Science Publishers, Inc., Lebanon, 9-22

Ruiz, M. A. 2009. El análisis de tetrazolio en el control de calidad de semillas. *Caso de estudio: cebadilla chaqueña. EEA INTA Anguil Argentinca (77)*, 1-19.

Semillas Paplotla SA de CV. Informe Anual, 2018

Zurita, W. Gómez, G. Atrián, E. Hernández, A. Granados, M. García, J. Salgado, R. Sánches, N. 2014. Establecimiento de un método eficiente de germinación *in vitro* y micropropagación del cirimo *(tilia mexicana* schlecht.) (tiliaceae). Polibotánica, 38: 129-144