In vitro establishment and conservation of *Aechmea bambusoides* L.B. Smith & Reitz, a threatened bromeliad species from Brazil [1]

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ABSTRACT

The present research aimed to develop a reliable technique for in vitro conservation of Aechmea bambusoides germplasm. A. bambusoides germplasm was successfully established in vitro through seeds obtained from field collection. The plant multiplication was achieved in a liquid Murashige and Skoog medium without growth regulators and its long term culture maintained at least for 1.5 year without losing their growth ability. Axillary shoots obtained from A. bambusoides in vitro grown plants were readily acclimatized in a substrate containing either Dicksonia sellowiana fiber (xaxim) or Eichhornia crassipes fiber (aguapé).

Key-words: in vitro establishment, germplasm conservation, bromeliads, acclimatization, Dicksonia sellowiana fiber, Eichhornia crassipes fiber

RESUMO

Conservação *in vitro* de *Aechmea bambusoides* L.B. Smith & Reitz, uma espécie de bromélia brasileira ameaçada de extinção

O objetivo do presente trabalho foi desenvolver uma técnica segura para conservação in vitro do germoplasma de *Aechmea bambusoides*. O germoplasma de *Aechmea bambusoides* foi estabelecido in vitro através de sementes obtidas de coleção ativa de campo. A multiplicação das plantas foi obtida em meio MS líquido sem regulador de crescimento e mantidas por um período longo de pelo menos 1,5 ano, sem a perda de sua capacidade de desenvolvimento. Durante esse período brotações axilares obtidas de plantas in vitro *Aechmea bambusoides* puderam ser prontamente aclimatizadas em um substrato composto tanto por fibra de *Dicksonia sellowiana* (xaxim) quanto por fibra de *Eichhornia crassipes* (aguapé).

Palavras-chave: estabelecimento in vitro, conservação de germoplasma, bromélias, aclimatização, fibra de *Dicksonia sellowiana*, fibra de *Eichhornia crassipes*

1. INTRODUCTION

Aechmea bambusoides L.B. Smith & Reitz is a threatened Bromeliad plant which grows in the states of Rio de Janeiro and Minas Gerais, Brazil (MENDONÇA and LINS, 2000). It is composed of light green spinulose foliage arranged in spiral configuration and a large open rosette which resembles a jar. Its long lasting blooms with brilliant multicolor inflorescence and its ornamental foliage makes it a desirable landscaping plant (PAULA and SILVA, 2001).

A. bambusoides natural population is found on remaining semideciduous Atlantic Forest located in private lands along Paraiba River Valley. Because of the private nature of these land holdings it is difficult to establish appropriate *in situ* conservation systems for these species. Furthermore the natural habitat is under constant pressure due to land clearing, mining, forest harvesting, or settlement by farmers. Therefore e*x situ* conservation appears to be the best approach for conservation of *A. bambusoides*.

Ex situ conservation of seeds are used for major crop plants such as cereals and grain legumes. In this case, seeds are dried slowly and carefully, packed in sealed containers, and placed in refrigeration under low humidity (PLUCKNETT and HORNE, 1992). It is expected that many seeds can be preserved under such conditions for prolonged period of time. However, Bromeliads produce recalcitrant seeds, which cannot be dried without rapid loss of viability and that are also short-lived when moist

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(ATLEE, 2004). The options for conservation of species producing recalcitrant seeds are either field collections or vegetative propagules stored using *in vitro* techniques (VILLALOBOS et al., 1991). Field collections in addition to being labor-intensive and expensive is geographically restricted and exposes the material to phytopathogens and environmental calamities, resulting in loss of genetic diversity (VILLALOBOS et al., 1991). Plant tissue culture has been used as a reliable tool in rapid clonal propagation of selected genotypes of several Bromeliaceae (CARNEIRO et al., 1999, HOSOKI and ASAHIRA, 1980, KOH and DAVIES JR., 1997), and has been quoted as a suitable method for the germoplasm preservation of this family.

The present investigation aimed to develop a technique to conserve *A. bambusoides* germplasm *in vitro*. In this paper we report a method to establish and maintain *in vitro A. bambusoides* germplasm. We further report these cultures can be maintained for at least 1.5 years, and they could be readily acclimatized during that period.

2. MATERIAL AND METHODS

The present investigation was conducted at Plant Cell and Tissue Culture Laboratory in the Plant Science Department of the Federal University of Vicosa, Brazil, and divided into four steps: *in vitro* culture establishment, *in vitro* plant multiplication, *in vitro* long term maintenance, and plant acclimatization.

2.1. In vitro culture establishment

In vitro culture of *Aechmea bambusoides* L.B. Smith & Reitz was started from seeds obtained from the Unit for Bromeliaceae Conservation and Research at Federal University of Vicosa, Brazil. The seeds were disinfested through immersion in alcohol 70% (v/v) for one minute, rinsed in sterile deionized water, and then immersed in a 2.5% (active chlorine) sodium hypochlorite solution with 0.01% (v/v) Tween 20 for 15 minutes under vigorous agitation. The establishment medium was composed by 50% salts and 100% organic compounds of Murashige and Skoog (1962) basal medium (MS) with no additions of growth regulators. The medium pH was adjusted to 5.7 and 8.0 g L^{-1} agar (Grupo Química, Brazil) was added as gelling agent. The medium was then disposed in 25 x 150 mm test tubes (10 ml per tube) and autoclaved for 20 minutes at 121ºC and pressure of 103 Kpa. After disinfestation, the seeds were rinsed three times in sterile deionized water and inoculated in the test tubes containing semisolid medium and incubated at 25-29°C under cool white fluorescent light with 16 hr photoperiod. The photosynthetically active radiation (PAR) at the level of the medium surface was 40µmol $m^{-2}s^{-1}$.

2.2. *In vitro* plant multiplication

To establish an appropriate multiplication medium five concentrations (0.0, 10.0, 20.0, 40.0, and 80.0 mM) of 6-benzyladenine (BAP) and two concentrations (50% and 100%) of MS salts were tested in a 5 x 2 factorial in a randomized complete block experiment with six replications. The media were also composed of full MS organic nutrients and had the pH adjusted to 5.7. Before autoclaving for 20 minutes at 121ºC they were disposed in 350 ml pickling jars (15 ml per jar). One plantlet per jar was inoculated in these liquid media. These plantlets were maintained floating on the medium, without aeration, in steady state for seven weeks under the environmental conditions described previously. The number of axillary shoots and the mean shoot length (mm) were measured per treatment and results were analyzed statistically using the System for Statistical Analysis (SAEG 9.1).

2.3. *In vitro* long term storage

Seeking *in vitro* long term maintenance of *A. bambusoides*, plantlets growing in appropriate multiplication medium were transferred to long term maintenance medium (LTMM). This medium was composed of 50% salts and 100% organic nutrients of MS medium. The medium pH was adjusted to 5.7 and dispensed in 350 ml pickling jars (15 ml liquid LTMM per jar) before autoclaving for 20 minutes at 121ºC. Only one plantlet per jar was held in this experiment. The jars were maintained in steady state in a room kept under environmental conditions described previously. Every three month each jar was refilled with 15 ml fresh sterile LTMM and after nine months the cultures were subdivided and transferred to new jars containing fresh LTMM.

2.4. Acclimatization

A. bambusoides shoots growing in LTMM were detached from mother plants and classified in two groups according to their height: H1 (20 to 40 mm) and H2 (41 to 60 mm). Two different substrates, *Dicksonia sellowiana* (Pr.) Hook fiber (xaxim), and *Eichhornia crassipes* (Mart.) Solms fiber (aguapé) were tested for acclimatization of these *in vitro* grown plants in a 2 x 2 factorial, in a randomized complete block experiment with five replications and 12 shoots per experimental unit. The shoots were planted in 50 ml styroblock plugs and maintained in a covered environment (50% shade cloth, SombriteÒ). The irrigation was provided on a daily basis and the fertilization on a weekly basis with 2.0 gL^{-1} PetersÒ (NPK 20-20-20) through foliar applications. After 60 days from transplanting the percentage of survival, the number of roots produced per plant, and the dry matter of plants were determined and the results were statistically analyzed using SAEG 9.1.

3. RESULTS AND DISCUSSIONS

3.1. *In vitro* culture establishment

A. bambusoides was successfully established *in vitro* using seeds as starting explants. The seed germination started 20 days after inoculation and in 30 days reached 99%. Less than 1% contamination was observed in this experiment indicating that the disinfestation method was sufficient to eliminate most of fungi and bacterial contaminants without affecting seed viability and thus rate of germination.

3.2. *In vitro* plant multiplication

The *in vitro* multiplication of *A. bambusoides* was successfully achieved in this experiment. The development of axillary shoots was observed in all media tested, independently of BAP concentrations or MS salt

concentrations or their combinations. However, BAP significantly increased the number of axillary shoots (figure 1.I). The highest number of shootings was observed in 10 mM of BAP, ca. 19.60 shoots per plantlet, and then decreased slowly as the BAP concentration was increased. The lowest number of axillary shoots was obtained in the absence of BAP, ca. 4.57 shoots per plantlet. Although lower than the numbers of shoots obtained in the presence of BAP, it indicates that *in vitro* multiplication of this species can be accomplished without any additions of growth regulators in the medium, which is an advantage concerning germplasm conservation, because plant growth regulators might increase the frequency of somaclonal variation in tissue culture, especially when used in high concentrations (PIERIK, 1987). Consequently, for *in vitro* multiplication of *A. bambusoides* in germplasm banks the use of medium with no BAP is recommended. However 10 mM of BAP may be used for mass production of plant stocks in commercial nurseries.

The axillary shoots obtained in the presence of BAP were shorter than those obtained without BAP (figure 1.II). The height of shoots grown in the presence of BAP was on average 2.5 times shorter than to those grown in the absence of BAP. Probably it was due to higher level of competition for space and nutrients among shoots induced by increase in the number of shoots in these treatments. Furthermore BAP concentrations higher than 10 mM also decreased the multiplication rates of *A. bambusoides*, suggesting that BAP might be phytotoxic to tissue culture of this species at this range. For instance the inhibitory effect of BAP was previously reported by KISS et al. (KISS et al., 1995) in pineapple (*Ananas comosus*) *in vitro* culture.

The number of axillary shoots was significantly higher in the media containing full MS salt than in the media with 50% MS salt (figure 2.I). Higher salt concentration in the medium also increased the height of shoots; consequently shoots obtained in full MS salt were 7.9 mm taller than obtained with 50% MS salt. However this difference was only observed in the absence of BAP being null in the presence of this growth regulator (figure 2.II).

3.3. *In vitro* long term storage

A. bambusoides culture has been maintained up to nine months in LTMM, without transferring to fresh medium. During this time new axillary shoots were formed

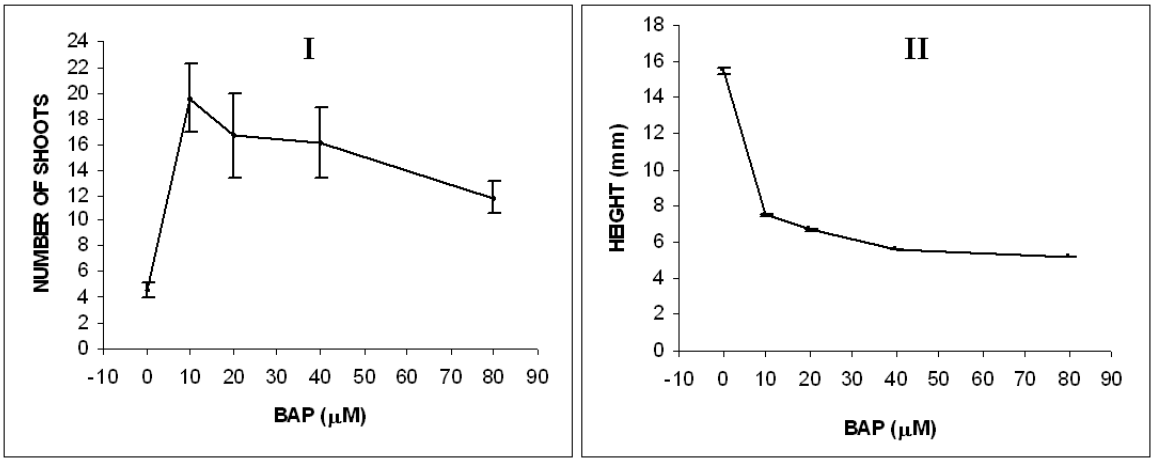

Figure 1. The effect of different concentrations of BAP on the number of axillary shoots (I) and height of axillary shoots (II) of *Aechmea bambusoides* growing in vitro. The bars represent standard error of the mean.

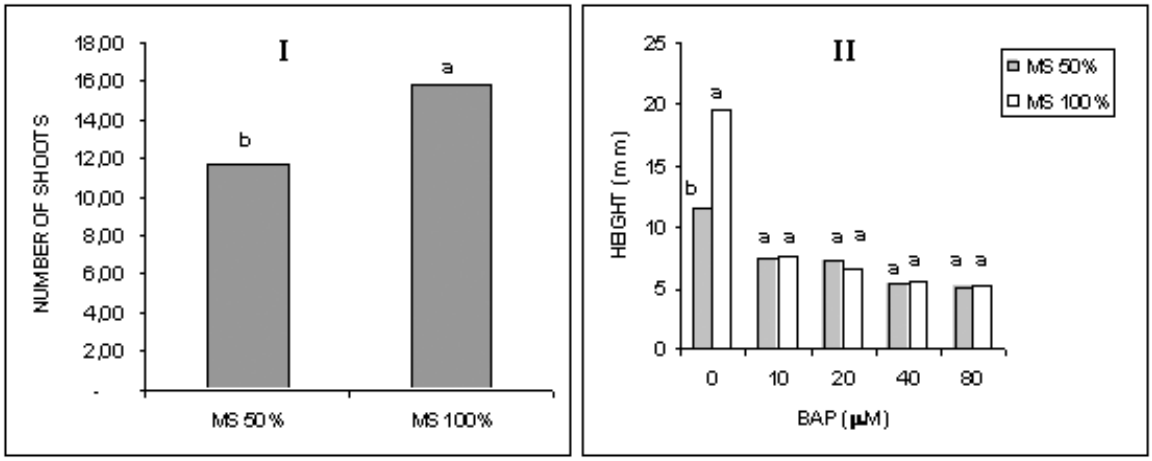

Figure 2. The effect of different MS salt concentrations on the number of axillary shoots (I) and height of axillary shoots (II) of *Aechmea bambusoides* growing in vitro. The means followed by different letters are significantly different at P<0.05 by Tukey's test.

from original explants which degenerated slowly as the new shoots grew. The development of these shoots was not synchronized, however at the end of this time most of them were 20 to 60 mm long. When these new shoots were detached from the mother plants and replicated to fresh LTMM, they repeated the growth pattern of their mother plants showing the feasibility of *in vitro* conservation of *A. bambusoides* germplasm. So far the long term culture has been maintained for more than 1.5 year without losing their ability to grow and their competency to regenerate in the conditions described here.

3.4. Acclimatization

The acclimatization of *A. bambusoides vitroplants* was successfully achieved in this experiment. More than 85% of *in vitro* grown plants survived the process in all treatments tested. Neither substrates nor heights of shoots significantly affected the rate of survival of the *in vitro* grown plants. However, at the end of acclimatization the taller *in vitro* grown plants developed into a more vigorous plants with higher dry matter than the shorter ones (figure 3.I), which has the potential to influence the development of adult plants. Therefore, 41 to 60 mm long *in vitro* grown plants is recommended for acclimatization. The shorter *in vitro* grown plants should be maintained *in vitro* until the proper height is achieved prior to acclimatization.

Acclimatized plants developed more roots in xaxim than in aguapé (figure 3.II), which might be due to a better aeration provided by xaxim in the acclimatization conditions of this experiment. However, the substrate did not influence the rate of survival or dry matter accumulation in plants, probably because roots in this species functions more as an anchoring structure than as a water and nutrients uptake structure. Moreover *A. bambusoides* is an epiphytic plant and its leaves are covered by plenty of trichomes from where water and nutrients can be absorbed (SMITH and DOWS, 1979).

Finally *D. sellowiana* from what xaxim is extracted is an endangered species (IBAMA, 1992) and its commercial exploitation has been prohibited in Brazil. *E. crassipes* (aguapé) on the other hand is an abundant source of fiber which can successfully substitute xaxim as a substrate for *A. bambusoides* acclimatization.

4. CONCLUSIONS

A. bambusoides can be easily established and maintained *in vitro* for a period longer than 1.5 year, without losing its ability to grow or its competency to regenerate. The *in vitro* multiplication of this species does not require the use of growth regulators, however in the presence of 10 mM BAP the multiplication rates was significantly increased. The acclimatization of *in vitro* grown plants was successfully achieved with more than 85% survivability. During acclimatization taller *in vitro* grown plants (41 to 60 mm) developed into a more vigorous and robust plants than shorter ones. Also, *E. crassipes* fiber (aguapé) can successfully substitute *D. sellowiana* fiber (xaxim) as a substrate for *A. bambusoides* acclimatization.

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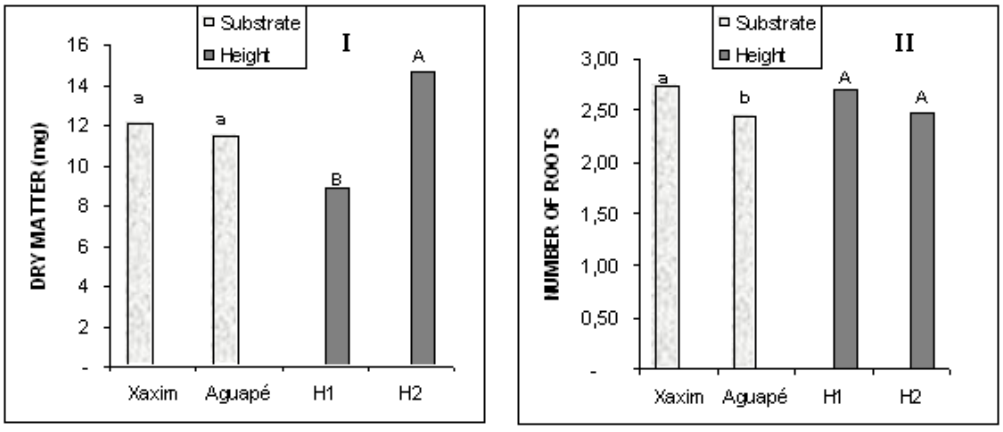

Figure 3. The effect of substrates and height of axillary shoots on dry matter (I) and number of roots (II) of Aechmea bambusoides acclimatized plants. H1: 20 to 40 mm in vitro grown plants. H2: 41 to 60 mm in vitro grown plants. The means followed by different letters are significantly different at P<0.05 by Tukey's test.

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