

## Chapter 13

### Use of growth retardants for banana (*Musa* AAA cv. Grand Naine) shoot multiplication in temporary immersion systems

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**Abstract:** Temporary immersion culture (TIS) offers several advantages over solid medium for banana shoot multiplication, e.g. TIS results in an increase in the multiplication rate and improves the quality of the plantlets. For a commercial application of this technique large vessels are required. When using 10-litre culture vessels an excessive growth of the shoots (leaves and pseudostem) was obtained, which limited the final number of shoots to be produced per flask and reduced the production capacity in the growth room. Labor costs also increased, since handling is more difficult when dividing and subculturing large shoots during the multiplication stage. The effect of growth retardants ancymidol (ANC), paclobutrazol (PBZ) and daminozide (DAM) in liquid shake cultures and TIS was investigated in order to reduce the size of the shoots and allow a better use of the space inside the culture vessel. In liquid shake cultures ANC and PBZ, independently of the tested concentrations, promoted bud cluster formation with reduced size and compact shape. Shoots multiplied with ANC or PBZ (2.5 mg l<sup>-1</sup>) after five subcultures, recovered their normal morphology after transfer to a hormone-free medium without growth retardants. However, during the acclimatization stage, plants multiplied in ANC (2.5 mg l<sup>-1</sup>) containing media showed reduced height in comparison with control plants and plants multiplied in PBZ and DAM containing medium. The application of PBZ and ANC in TIS (1-litre flasks) stimulated bud proliferation. Both compounds were also effective in controlling the excessive growth of the shoots and in inducing the formation of compact bud clusters. Shoots multiplied in TIS in presence of PBZ (2.5 mg l<sup>-1</sup>) were successfully transferred to semisolid or liquid rooting media in traditional culture vessels or TIS. The developed protocol was further scaled up in 10-litre TIS vessels.

**Key words:** ancymidol, daminozide, paclobutrazol, propagation

*Abbreviations:* ANC – ancymidol; 6-BAP – 6-benzylaminopurine; DAM – daminozide; PBZ – paclobutrazol; TIS – temporary immersion system

## 1. Introduction

A broad commercial propagation of *Musaceae* by direct organogenesis (axillary shoot proliferation) has been limited due to the high costs of production, which is a result of the high number of manual operations needed. Additionally, the use of semisolid culture medium and the use of low capacity vessels limit the possibility to automate or semi-automate *in vitro* propagation processes (Ziv, 1990).

The development of more efficient protocols based on the use of liquid culture medium in some or all the stages of micropropagation can reduce the required manipulations and thus can diminish the costs of *in vitro* propagation. Temporary immersion system (TIS) is an accessible technology that allows the partial automation of some steps of *in vitro* culture with major facility for scale up; increasing the biological and productive efficiency of the propagated material without the collateral effects caused by static liquid culture medium namely hyperhydricity and hypoxia (Alvard et al., 1993; Teisson et al., 1996; Lorenzo et al., 1998; Escalona et al., 1999; Jiménez et al., 1999; Etienne and Berthouly, 2002).

Banana shoot multiplication in TIS was first described by Alvard et al. (1993), who used modified Nalgene filtration units as culture vessels and afterwards RITA® has been successfully used by many laboratories. However, the commercial application of the technique requires larger vessels (Jiménez et al., 1999).

When scaling up from RITA to 10-litre twin flask system some problems arose, e.g. an excessive growth of the shoots (leaves and pseudostem), which limited the final number of shoots per flask reducing the production capacity of the growth room. Labor costs also increase, since handling is more difficult when dividing and subculturing large shoots during the multiplication stage. The addition of growth retardants to the culture medium to reduce the size of the shoots might be a solution to overcome these problems. Growth retardants already have been successfully used to inhibit shoot length and to promote bud clusters formation in several species including bananas (Ziv, 1992; Opatrná et al., 1997; Ziv et al., 1998; Escalona et al., 1999). The aim of this study was to evaluate the effect of growth retardants ancymidol (ANC), paclobutrazol (PBZ) and daminozide (DAM) in liquid cultures and TIS in order to reduce the size of the banana shoots and allow a better use of the space inside the culture vessel.

## 2. Materials and methods

### 2.1 *Plant material, culture media and culture conditions*

Shoots from banana (*Musa* AAA cv. Grand Naine) were established and multiplied according to the conditions and procedures described by Orellana (1994). For shoot multiplication the MS medium (Murashige and Skoog, 1962) was used, supplemented with 1.0 mg l<sup>-1</sup> of thiamine, 4.0 mg l<sup>-1</sup> of 6-BAP and 3.0% (w/v) sucrose. The rooting culture medium had the same composition, but no 6-BAP. The pH was adjusted to 5.8 before sterilization at 121°C and 1.2 kg cm<sup>-2</sup>.

The cultures were kept in a growth room with natural light at a temperature of 27 ± 2°C. Liquid cultures were shaken on a rotary shaker at 100 rpm (250 ml Erlenmeyer flasks). The immersion frequency in TIS (1- and 10-litre culture vessels) was 1 minute every 6 hours. Cultures on semisolid medium were transferred to fresh medium every 21 days. In liquid shake cultures and TIS, the medium was substituted every 15 days.

Rooted plants were transferred to the acclimatization phase in trays of poly-foam with 70 orifices (120 cm<sup>3</sup> each) with a substrate composed of 75% humus and 25% zeolite (v/v). The temperature ranged between 25-30°C, under 50% shade and the irrigation was provided by micro-sprinklers with a duration and frequency of two minutes every four hours.

### 2.2 *Shoot multiplication in liquid shake cultures*

A first series of experiments were performed in order to evaluate the effect of growth retardants (ANC, DAM and PBZ) at four concentrations (0.0, 1.0, 2.5 and 5.0 mg l<sup>-1</sup>). Five explants were inoculated per flask containing 20 ml of liquid multiplication medium and five flasks were used per treatment. The formation of clusters, the number of shoots per explant, the final weight (total weight of the explant at the end of the culture time), the discarded weight (weight of the leaves and tissue rejected) and the useful weight (weight of the sectioned shoots used for the following subculture) were evaluated.

A comparative study was carried out during the elongation/rooting stage and the acclimatization stage on the morphology of plants multiplied during five subcultures in ANC or PBZ (2.5 mg l<sup>-1</sup>) containing media. Plants from standard semisolid medium and liquid shake medium were included as controls. After the fifth subculture, individual shoots were separated and transferred to semisolid rooting medium. Five shoots were placed per flask using a total of 20 flasks per treatment. The length and width of leaf 2,

length of the petiole of leaf 2, diameter of the pseudo stem, number of leaves and roots were evaluated.

For the acclimatization stage 70 plants were evaluated 45 days after transfer to *ex vitro* conditions and the same morphological parameters described previously for the elongation/rooting stage were measured.

### 2.3 *Shoot multiplication in TIS*

An experimental unit with 1-litre twin flask TIS according to Jiménez et al. (1999) was used. Twentyfive bud clusters multiplied with 2.5 mg l<sup>-1</sup> of ANC or 2.5 mg l<sup>-1</sup> of PBZ in liquid shake medium, were inoculated per TIS unit. Each TIS unit contained 500 ml of multiplication medium supplemented with 2.5 mg l<sup>-1</sup> ANC or PBZ. A control treatment was included with shoots cultivated in standard multiplication medium. Two TIS units were used per treatment and the experiment was replicated twice. The number of shoots/explant, the final weight, discarded weight and the useful weight of the shoots were evaluated. Bud clusters multiplied in TIS with 2.5 mg l<sup>-1</sup> of PBZ, were transferred to semisolid and liquid rooting medium.

All the statistical analyses were done with the program "Statistix" (Copyright © 1996 Analytical software) version 1.0 for Microsoft Windows.

## 3. Results and discussion

### 3.1 *Effect of ancymidol, daminozide and paclobutrazol on shoot multiplication in liquid shake cultures*

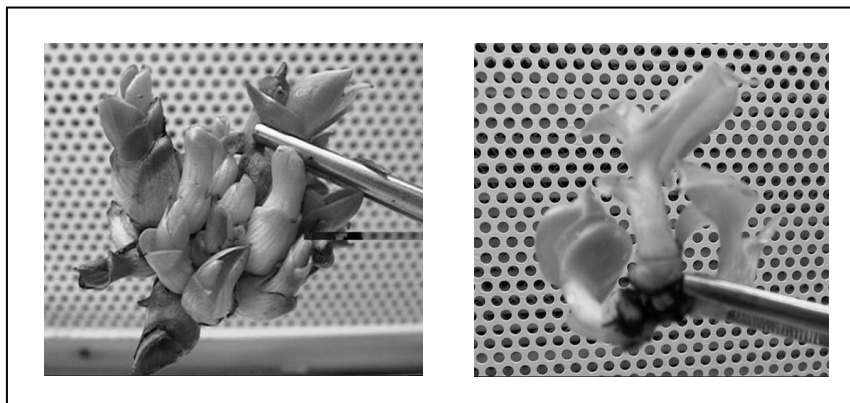
ANC and PBZ, independently of the concentration tested, proved to be effective in reducing the size of the shoots and in inducing the formation of compact bud clusters (Figure 1). The formation of compact bud clusters was achieved in all explants treated with ANC and PBZ; whereas all the explants on DAM containing medium showed similar growth and development to the control cultures without growth retardants. Bud clusters were characterized by agglomerates of small and compact shoots with a remarkable reduction of longitudinal growth of the pseudostem, and an increase in their thickness of up to 1.0 cm in diameter. The leaves were poorly developed, folded and compact, which differed in size and development from the shoots grown on the control medium or DAM containing media. The latter, developed thinner pseudo stems with one to three completely developed and expanded leaves.

According to Ziv (1990), the absence of growth retardants allowed the leaves of *Gladiolus* to continue their growth and elongation in liquid culture

medium; whereas in the presence of PBZ and ANC, a reduction in the number and length of the leaves was observed and the formation of clusters was obtained. These results confirm that the addition of growth retardants induces a shoot morphology characterized by the decrease in length of the stems and leaves.

Both compounds also stimulated bud proliferation, increasing the number of shoots produced per inoculated explant, 5.4 and 4.6 shoots for ANC and PBZ containing media respectively, with significant statistical differences with the control medium which contained only BAP (2.4 shoots per explant) (Figure 2). No statistical differences were found between all tested concentrations of both growth retardants. The significant increase in the number of shoots per explant when using ANC and PBZ could also be associated with the inhibition of gibberellin biosynthesis, which suppress the growth and dominance of the apical bud, favoring or stimulating the shooting of axillary buds (Grossmann, 1990).

DAM is described in the literature as a growth retardant in plants and though it is demonstrated that it does not exercise inhibitory effect by interruption of the synthesis of gibberellins (Smith et al., 1991), it could interfere in the action of these and favor the action of the cytokinins. In this way, an increase in the number of shoots could be obtained without morphological changes of its normal structure.



*Figure 1:* Bud clusters obtained from ANC- or PBZ-containing medium (left) and elongated shoots developed on DAM-containing medium or control medium free of growth retardants (right).

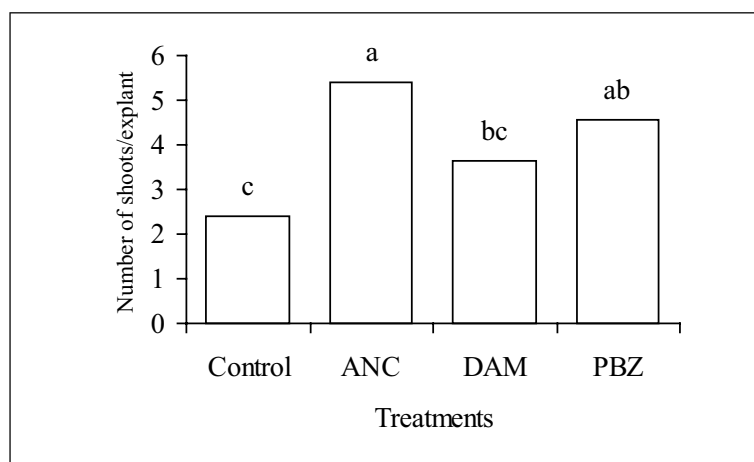


Figure 2: Effect of growth retardants (ANC, DAM and PBZ) on banana shoot multiplication in shaken liquid media.

Different letters represent significant differences according to Sheffe's test,  $p < 0.05$ .

Supplementing the medium with the different growth retardants did not influence the final weight of the shoots. The final weight is composed of 'discarded weight' and the 'useful weight' (including meristematic tissue) of the shoots; these being complementary, showed an inversely proportional behavior. For the discarded weight of the shoots, the ANC and PBZ presented differences with respect to DAM and the control. The useful weight of the shoots showed a similar behavior, but inversely related (Table 1). This behavior is due to the fact that the weight of all shoots increased. However, they differed in the discarded weight and useful weight, since the shoots from the control and DAM medium increased their weight because of the growth and elongation of the stems and leaves, unlike the shoots exposed to ANC and PBZ which increased in weight due to the formation of new shoots. These differences are observed clearly in the useful weight, since both ANC and PBZ showed the greatest weight due to new shoots and not necessarily due to the growth of leaves and stems that are eliminated with the handling of the explants for the following subculture.

Ziv et al. (1998) reported a gain in total weight of 35.2 g when inoculating 10 g bud clusters of cv. Grand Naine in liquid medium in agitation with ANC, obtaining 60.7% of meristematic tissue (useful tissue) and 39.3% of expanded leaves and necrotic tissue after 25 days.

Shoots multiplied in ANC and PBZ containing media after five subcultures, recovered their normal morphology after transfer to a hormone free medium and started to form roots (Figure 3), which reaffirms there is no

necessity to apply auxins to induce rooting in cv. Grand Naine; seemingly the endogenous concentration of this regulator is sufficient to restore the internal balance of growth regulators, when cytokinins are absent from the culture medium (Sandoval et al., 1999).

Additionally, it was possible to restore the normal length of the pseudostems and new leaves by elimination of the growth retardants and BAP as reported by Ziv (1989, 1992) for *Gladiolus*, ferns and bananas. This is in contrast with the results of Escalona (1999) in pineapple and Lorenzo et al. (1998) in sugarcane, who applied gibberellins to obtain plants with a suitable size for acclimatization. Such results indicate that there is no residual effects of the growth retardants ANC and PBZ, in the concentration used ( $2.5 \text{ mg l}^{-1}$ ), on the morphology of the plants. However, these cultures were only subjected to five consecutive cycles.

When plants were transplanted to the greenhouse, differences were observed in plant height (Table 2). Those plants coming from ANC containing media during the multiplication stage showed a reduced size compared to plants from control in semisolid or liquid medium and from plants multiplied in PBZ containing medium. This resulted in an extended acclimatization stage for the plants multiplied in ANC containing media.

The parameters evaluated indicated in field experiments that growth retardants present during five consecutive multiplication subcultures are not an apparent source or cause of somaclonal variability in plants of cv. Grand Naine. However, field evaluations are the most trustworthy to determine somaclonal variants, since the plants have expressed their phenotypic and phenologic potential (Sandoval et al., 1997). Hence the field studies will be continued with the plants coming from propagation systems with these growth retardants (ANC and PBZ).

### 3.2 *Effect of ancymidol and paclobutrazol on shoot multiplication in TIS*

Once the efficiency of ANC and PBZ to control excessive growth of the shoots was determined and the possibility to recover normal plants in liquid shake culture was demonstrated, a second group of experiments was conducted in 1-litre TIS vessels. The addition of ANC and PBZ also induced the formation of bud clusters in TIS (Table 3). As in liquid shake cultures ANC and PBZ promoted shoot multiplication with significant statistical differences compared to the control (Figure 4). Differences were also observed for the discarded and useful weight of the shoots in TIS, while the final weight of the shoots did not show statistical differences (data not given in Table 3).

*Table 1:* Influence of ANC, DAM and PBZ on the discarded and useful weight of banana shoots multiplied in shaken liquid medium

Growth retardants	Discarded weight (g)	Useful weight (g)
ANC	0.80 b	0.98 a
DAM	1.03 a	0.54 b
PBZ	0.70 b	0.91 a
Control	1.16 a	0.48 b
$\bar{x} \pm \text{s.e.}$	$0.91 \pm 0.05$	$0.73 \pm 0.029$

Different letters in a row represent significant differences according to Sheffe,  $p < 0.05$ .

*Table 2:* Effect of growth retardants (ANC and PBZ,  $2.5 \text{ mg l}^{-1}$ ) during the multiplication stage on plant height after 10 days at the acclimatization stage

Growth retardants during the multiplication stage	Plant height (cm)
ANC (liquid)	4.92 b
PBZ (liquid)	5.60 a
Control (liquid)	5.70 a
Control (semisolid)	5.65 a
$\bar{x} \pm \text{s.e.}$	$5.46 \pm 0.14$

Different letters in a row represent significant differences according to Tukey's test  $p < 0.05$ .

*Table 3:* Influence of ANC and PBZ on the discarded and useful weight of banana shoots multiplied in 1-litre TIS vessels

Growth retardants	Discarded weight (g)	Useful weight (g)
$2.5 \text{ mg l}^{-1}$ of ANC	1.46 b	1.39 a
$2.5 \text{ mg l}^{-1}$ of PBZ	1.40 b	1.55 a
Control	2.43 a	0.66 b
$\bar{x} \pm \text{s.e.}$	$1.63 \pm 0.10$	$1.31 \pm 0.08$

Different letters in a row represent significant differences according to Tukey's test,  $p < 0.05$ .



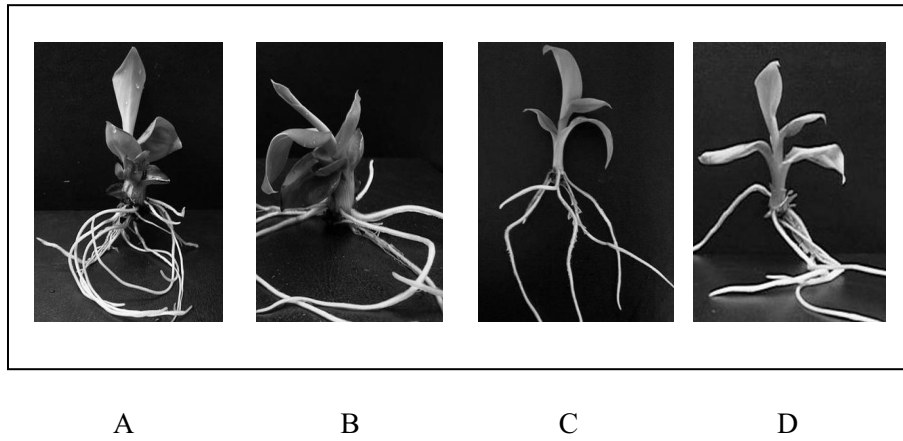


Figure 3: Plants obtained at the end of the elongation/rooting stage, after 15 days in a hormone-free medium. A: 2,5 mg l<sup>-1</sup> ANC; B: 2,5 mg l<sup>-1</sup> PBZ; C: Control in liquid medium; D: Control in semisolid medium.

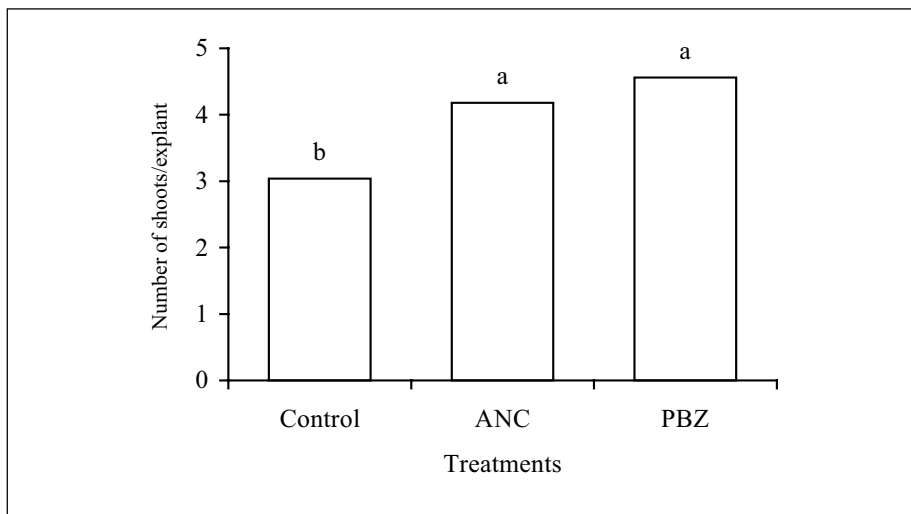


Figure 4: Shoot multiplication in 1-litre TIS on ANC and PBZ (2.5 mg l<sup>-1</sup>) containing media. Different letters represent significant differences according to Tukey's test,  $p < 0.05$ .

The discarded weight and the useful weight of the shoots indicate that gain in the final weight is caused by the greater number of shoots in presence of ANC and PBZ, while the gain in weight of the control was due to the unwanted fast growth of the leaves and stem, hence the reason why this treatment resulted in the greatest discarded weight and the lowest useful weight, which determined the statistical differences of this variable with respect to the ANC and PBZ treatments.

Lorenzo et al. (1998) used PBZ in micropropagation of sugarcane in TIS, obtaining plants in the field similar to those micropropagated by conventional methods, however, TIS resulted in a reduction of 46% of the production costs. In pineapple, Escalona (1999) developed a semi-automated system more efficient than the conventional micropropagation based on the use of PBZ in TIS, which reduces the production costs by 66.7%. Besides, the plants showed better *ex vitro* growth and development in comparison to the plants obtained by conventional micropropagation.

Shoots multiplied in TIS with PBZ ( $2.5 \text{ mg l}^{-1}$ ) were singulated and subcultured for rooting in hormone-free medium, either semisolid or liquid medium. After 15 days plants were ready for acclimatization.

The protocol developed was suitable for scale up in 10-litre TIS vessels, using a two-stage procedure. First shoots were multiplied in PBZ and BAP containing medium during 4 weeks and secondly, inside the same culture vessel, the multiplication medium is replaced by a hormone free medium to promote shoot elongation and rooting (Figures 5 and 6).

#### 4. Conclusion

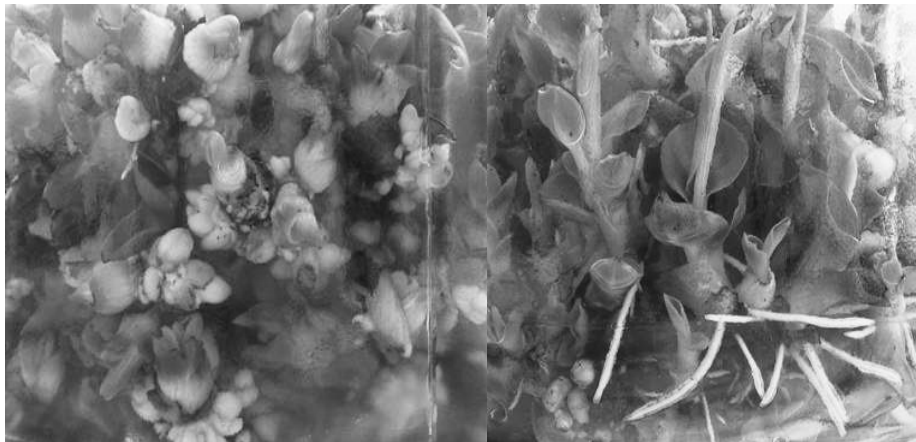
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The addition of ancymidol (ANC) and paclobutrazol (PBZ) reduced the size of banana shoots multiplied both in liquid shake cultures and TIS. Both compounds also stimulated bud proliferation and increased the number of shoots produced per inoculated explant. Shoots multiplied in ANC and PBZ containing media, either in liquid shake cultures or 1-litre TIS, recovered their normal morphology after transfer to a hormone free medium, which indicate that there is no residual effects of the growth retardants ANC and PBZ on the morphology of the plants. Only the plants multiplied in ANC containing media showed a reduced size and an extended acclimatization

period compared to plants from control in semisolid or liquid medium and plants multiplied in PBZ containing medium.



*Figure 5:* Scale up of banana shoot multiplication in 10-litre TIS vessels (left) and rooting (right).



*Figure 6:* Banana shoots multiplied in 10-litre TIS in PBZ (2.5 mg l<sup>-1</sup>) containing medium (left) and subsequent elongation/rooting in a hormone-free medium (right).

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