**IPEF n.48/49, p.117-125, jan./dez.1995**

**TRABALHO DE PESQUISA /** RESEARCH PAPER

# IN VITRO PROPAGATION OF BLACK WATTLE (*Acacia meamsii* De Wild)

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**ABSTRACT** - The in vitro propagation of black wattle (**Acacia mearnsii** De Wild) was obtained from 9 months, old seed propagated plants. The medium used was MS (Murashige & Skoog, 1962) with half the concentration of inorganic salts. Nodal segments measuring 5 mm long formed induced shoots with growth higher than 10 mm in the presence of BA at 0.05 or 0.1 mg.l$^{-1}$ and/or BA and IBA at 0.05 mg.l$^{-1}$ respectively at 7 days of culture. The higher proliferation of shoots (3.51 shoots/explant) was obtained in the presence of BA at 3.0 mg.l$^{-1}$ and IBA at 0,05 mg.l$^{-1}$. The reduction in the leves of BA (0.5 to 0.05 mg.l$^{-1}$) promoted shoots elongation. Up to 4 shoots/explant measuring 10 to 20 mm were obtained at 30 days of culture. There was some necrosis in the shoot tips in the multiplication and elongation phases after 30 days and until 30 days, respectively. In the presence of IBA at 1.0 mg.l$^{-1}$, some calli were formed at the shoots base, with no induction of roots.

**RESUMO** - A propagação in vitro de acácia negra (**Acacia meansii** De Wild.) foi obtida a partir de plantas, com 9 meses de idade. Foi utilizado o meio de cultura MS (Murashige & Skoog, 1962) com as concentrações dos sais inorgânicos reduzidas à metade. Segmentos nodais medindo 5 mm de comprimento formaram gemas com crescimento superiora 10 mm de comprimento em presença de 0,05 ou 0,1 mg.l$^{-1}$ BA e/ou 0,05 mg.l$^{-1}$ BA e AIB, respectivamente, aos 7 dias de cultura. Maior proliferação de brotos (3,5 brotos/explante) foi obtido com a suplementação de 3,0 mg.l$^{-1}$ BA e 0,05 mg.l$^{-1}$ AIB. A redução dos níveis de BA (0,5 para 0,05 mg.l$^{-1}$) favoreceu o alongamento dos brotos. Foram obtidos até 4 brotos/explante com 10 a 20 mm de comprimento, aos 30 dias de cultura. Houve presença de necrose nos ápices dos brotos nas fases de multiplicação e alongamento após 30 dias e até 30 dias, respectivamente. Na presença de 1,0 mg.l$^{-1}$ AIB, verificou-se formação de "calos" na base dos brotos, sem indução de raízes.

## INTRODUCTION

Black wattle (**Acacia mearnsii** De Wild), native plant from Australian, is an economically important forest species in Brazil. It is characterized by fast growth and multiple uses, including pulp, fuelwood and timber production. However, it is cultivated mostly in the State of Rio Grande do Sul for tannin production, which places Brazil as the second leading world tannin exporter.

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Currently, genetic improvement programs for black wattle are in their early stages of development. Moreover, this species is propagated by seed which makes these programs very slow. Conventional techniques of vegetative propagation aimed to accelerate this process producing, so far, variable results (SHERRY, 1971; ASSIS et al., 1993).

In vitro propagation techniques offer a mean for rapid clonal propagation in order to achieve faster genetic improvement. Research involving plant regeneration by using this technique has not been reported for black wattle with success (ASSIS et al., 1993). Some studies have been done with the following species: **Acacia koa** (SKOLMEN & MAPES, 1976); **A. senegal** (DAVE et al., 1980; BADJI et al., 1992); **A. nilotica** (MATHUR & CHANDRA, 1983); **A. albida** (DUHOUX et al., 1985; CASSAMADIA & DUHOUX, 1992) and **A. ligulata** (WILLIAMS et al., 1985).

The aim of this study is regenerate black wattle through the establishment of in vitro propagation technique.

## MATERIAL AND METHODS

### Source of material

Seed propagated plants at 9 months old were used. The seeds were collected from selected trees at the TANAGRO S.A. plantations, in the city of Montenegro, State of Rio Grande do Sul -Brazil.

The plants were maintained in pots of 20 I capacity containing substrate constituted by 70% soil and 30% vermiculite of medium granulometry, with 50 g of NPK (8:17:6).

### Pretreatment, diseases and nutrition control of plants

The plants were maintained during 2 months in a greenhouse with 50% of light intensity. It was applied weekly a Benomyl solution (0,5 $g.l^{-1}$), with daily irrigation at the plants base. It was applied every 15days a solution of commercial fertilizer NPK (25:15:10) + micronutrients (1 $g.l^{-1}$).

### Desinfectation

It was observed in preliminary experiments that shoot from apical shoots and nodal segments, extracted from lateral branches of the plant, presented plagiotropic growth in vitro. Meanwhile, apical shoots and/or nodal segments excised from the plant tip presented orthotropic growth (non published data). Thus, the apical branch was used measuring about 50 mm. The defoliated branches were immersed into Benomyl solution (0.5 $g.l^{-1}$), followed by the immersion into commercial detergent solution at 3% (v/v), during 15 minutes, respectively. Under aseptic conditions, the branches were maintained during 15 minutes under shaking, in NaOCl solution at 1% (v/v), in the presence adding of Tween 20 at 0.01% (v/v). Then, they were washed three times in bidistilled and sterilized water.

### Culture medium

The culture medium used was MS (Murashige & Skoog, 1962) with half the concentration of inorganic salts with the supplements of ($mg.l^{-1}$): adenine (80.0), thiamine

(1.0), piridoxine (0.5), nicotinic acid (0.5), glycine (2.0), myo-inositol (100.0), sucrose (30000.0) and "Difco Bacto" agar (60000.0) added after adjusting the pH.

The culture medium was sterilized in autoclave at 121°C under 1.05 kg/cm$^2$, during 15 minutes.

**Inoculation of explants**

Nodal segments measuring 5 mm long were inoculated in culture tubes (25 x 150 mm) with 10 ml of medium, sealed with cotton plugs (1 explant/tube).

The cultures were maintained the first 7 days under light of 250 lux and 1000 lux after that. The explants were transferred at 10 days of culture to shoot induction medium.

**Shoots induction**

Combinations of growth regulators were tested: BA (0.05; 0.01 mg.l$^{-1}$) + IBA (0.0; 0.01; 0.05 mg.l$^{-1}$). Each explant was cultured in tubes with 10 ml of medium sealed with cotton plugs.

The shoots growth was evaluated at 7 and 14 days using classes of height: 1 = 5 to 10 mm, 2 = > 10 to 15 mm and 3 = > 15 to 20 mm.

**Shoots multiplication**

Combination of growth regulators was tested: BA (2.0; 3.0; 4.0 mg.l$^{-1}$) + IBA (0.0; 0.05; 0.1 mg.l$^{-1}$). Shoots were used measuring about 15 mm from the shoot induction phase cultured in medium containing BA and IBA at 0.05 mg.l$^{-1}$ respectively during 15 days.

Each explant was cultured in a 250 ml container sealed with polypropylene tap containing 25 ml of medium. The number and height of shoots were evaluated at 30 and 40 days.

**Shoots elongation**

Shoot proliferation was obtained in medium containing BA (3.0 mg.l$^{-1}$) and IBA (0.05 mg.l$^{-1}$) at 30 days. Shoot clusters with no explant sectioning were transferred to the medium with supplements of BA (0.0; 0.05; 0.1; 0.5 mg.l$^{-1}$). Containers and quantities of medium / container were the same as cited in the item Shoots multiplication. Each container contained 2 explants. The growth and vigor of shoots were evaluated at 30 days.

**Rooting of shoots**

Shoots (approximately 15 mm long) from the elongation medium (0.05 mg.l$^{-1}$ BA) cultured during 30 days were individually sectioned and transferred to medium with no adenine and with IBA (1.0 mg.l$^{-1}$). Containers and quantities of medium / container were the same as mentioned in the item Shoots multiplication. Each container contained 5 explants. Cultures were maintained 5 days in the dark followed by 10 days under light (1000 lux). Root formation and shoot vigor were evaluated.

## Growth conditions

Cultures were maintained at temperature of 25°C ± 1°C placed under 16 hour photoperiod (GRO-Lux Sylvania FUOT 12/GRO bulbs and Philips TL 40 W/54-RS fluorescent bulbs with 1000 lux).

## Statistical design

For the items Shoots induction and Shoots multiplication, each treatment had 3 replications with 20 explants each, placed in a randomized design, in factorial scheme. The effect of the treatments on the studied variables were evaluated through analysis of variance and Tukey's range test (1 %).

For the item Shoots elongation each treatment had 2 replications with 10 explants each. For the item Rooting of Shoots, 2 replication with 15 explants each were used. There was no statistical analysis for these items.

# RESULTS AND DISCUSSION

## Explants inoculation and shoots induction

Preliminary experiments showed that the rate of contamination may be reduced from 80% to 10% reducing explant size from 10 to 5 mm, long without effects on the shoots induction potential. Nobal segments obtained from the upper part of seedling were more effective for the shoots induction than apical shoots, both in MS medium with adenine (80.0 $mg.l^{-1}$). Apical shoots presented lower surviving and higher oxidation while nodal segments presented shoots up to 5 mm long at 14 days of culture (non published data).

The use of MS half strength plus BA (0.05 or 0.1 $mg.l^{-1}$) allowed the obtention of shoots with growth higher than 10 mm at 7 days of culture. It was possible to obtain shoots with growth higher than 15 mm at 14 days in medium with BA and IBA at 0.05 $mg.l^{-1}$ respectively. (Figure 1). The increase of BA to 0.1 $mg.l^{-1}$ in the presence of auxin allowed the inhibition of shoots growth (FIGURE 1) but did not affect the green color of the shoots observed in all treatments at 14 days of culture.

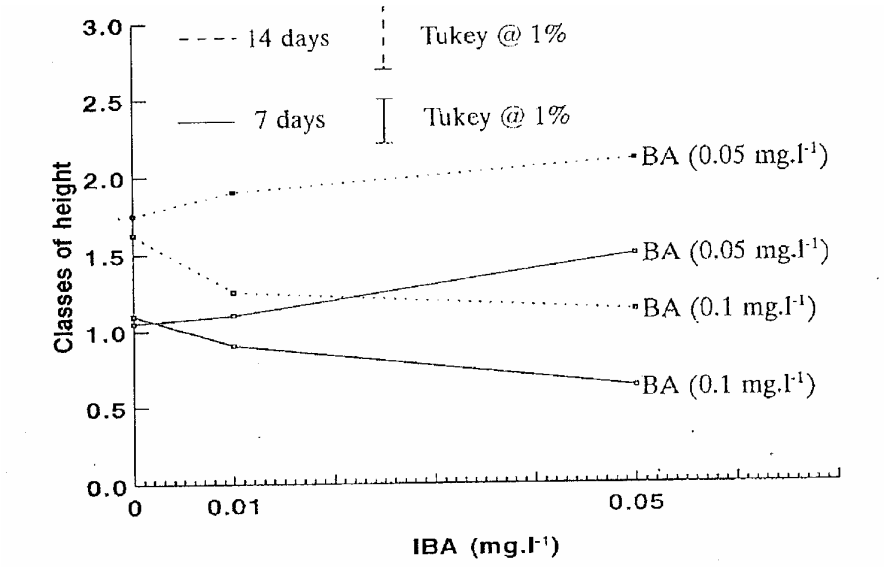


**FIGURE 1. Effect of BA and IBA concentrations on the shoots height growth of black wattle at 7th and 14th days of culture.**

Initial growth and development of shoots was affected by the medium composition. Reduction in the inorganic salts and presence of growth regulators allowed the growth of shoots in a short period of time. This means that the species may be established in medium of lower concentration of total ions, close to 45 mM, e.g WPM (LLOYD & McCOWN, 1980), GONÇALVES (1980) and JADS (CORREIA, 1993).

**Shoot multiplication**

Preliminary experiments, using medium cited in the item Culture medium, showed that BA at 2.0 $mg.l^{-1}$ increased shoot number (non published data). The use of BA at 3.0 $mg.l^{-1}$ + IBA at 0.05 $mg.l^{-1}$ induced the highest mean number of shoots / explant (3.51). However, there was no statistical difference for the use of BA at 2.0 $mg.l^{-1}$ (3.35 shoots / explant) at 30 days (FIGURE 2). Statistical differences among the mean number of shoots in these treatments were observed at 40 days (FIGURE 3).

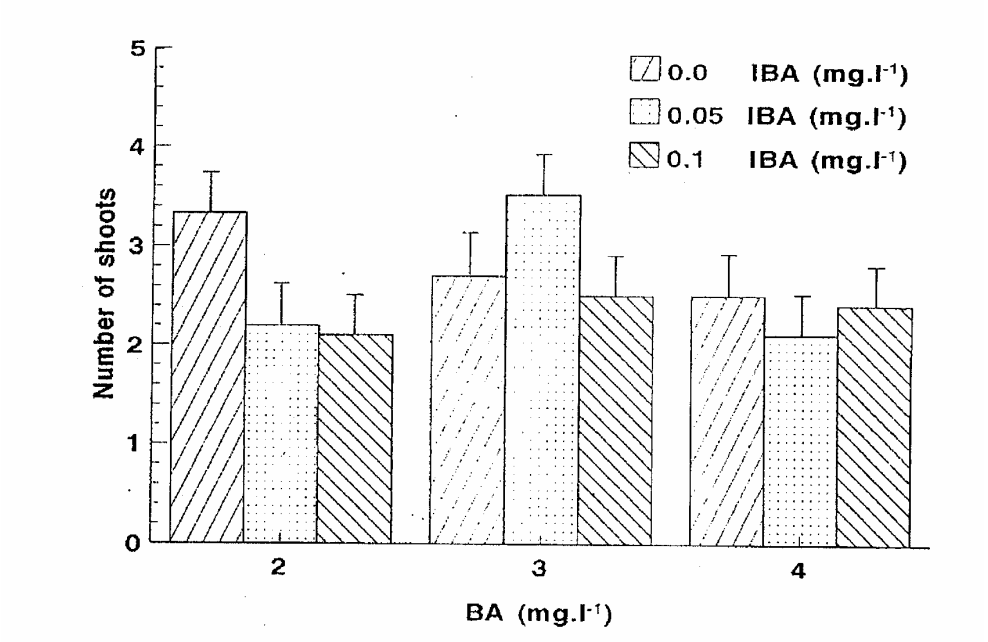


**FIGURE 2. Effect of BA and IBA concentrations on shoot multiplication (number) of black wattle at 30th day. Vertical lines represent Cl (p = 0.05).**

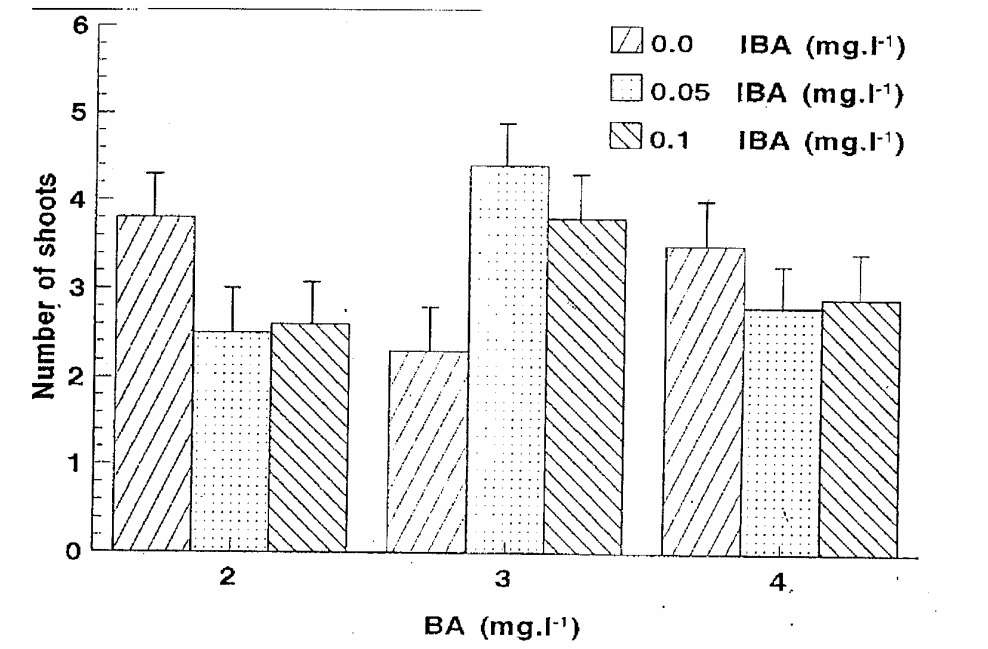


**FIGURE 3. Effect of BA and IBA concentrations on the number of shoot multiplication of black wattle at 40th day. Vertical lines represent Cl (p = 0.05).**

However, cultures maintained longer than 30 days showed necrosis in the tips of some shoots in all treatments. Factors connected to genetic variability, nutritional balance, environment and/or micro-environment conditions may have contributed for this result. According to SHA et al. (1985) apical necrosis in shoots may be related to a mineral nutrients deficiency, e.g., calcium, resulting from the high levels of humidity in the container micro-environment.

However, the system used for sealing the container allowed, apparently, neither accumulation of water vapor, nor explants with vitrification characteristics. It could be causing dehydration of the medium during the time of culture. Thus, it would contribute for the decreasing in the ions assimilation by the explant.

In culture of **Actinidia deliciosa** cultured in MS medium the pH decreased in the first 15 days and all elements were absorbed during 30 days being N and P practically exhausted during this period (MEZZETTI, 1991).

Considering the higher shoot growth in the first 7 days in the phase of shoots induction, it may probably occur a higher shoot growth and development in MS half strength during the multiplication in equivalent period. This suggests the realization of subcultures in a shorter period of time. The shoots multiplication rate could be increased and the shoots obtained with higher potential for the following phases of the system.

The use of BA (2.0 $mg.l^{-1}$) allowed the obtention of higher shoots while shorter shoots were obtained with BA at 4.0 $mg.l^{-1}$ (FIGURE 4 and 5) at 30 and 40 days of culture.

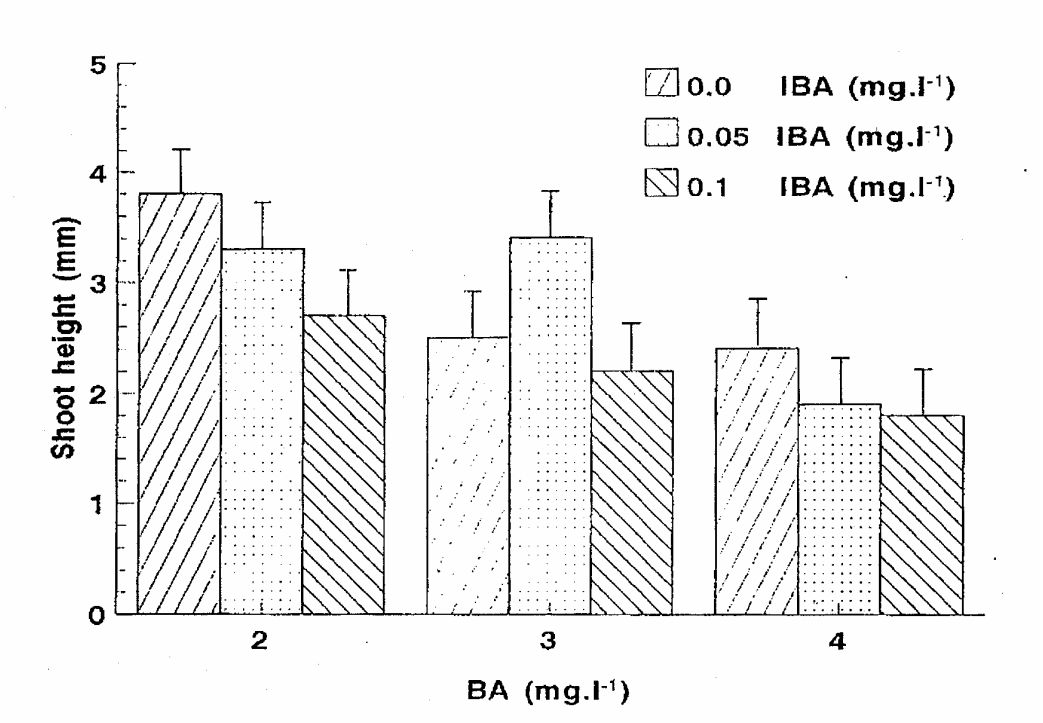


**FIGURE 4. Effect of BA and IBA concentrations on shoots height growth of black wattle at 30th day. Vertical lines represent Cl (p = 0.05).**

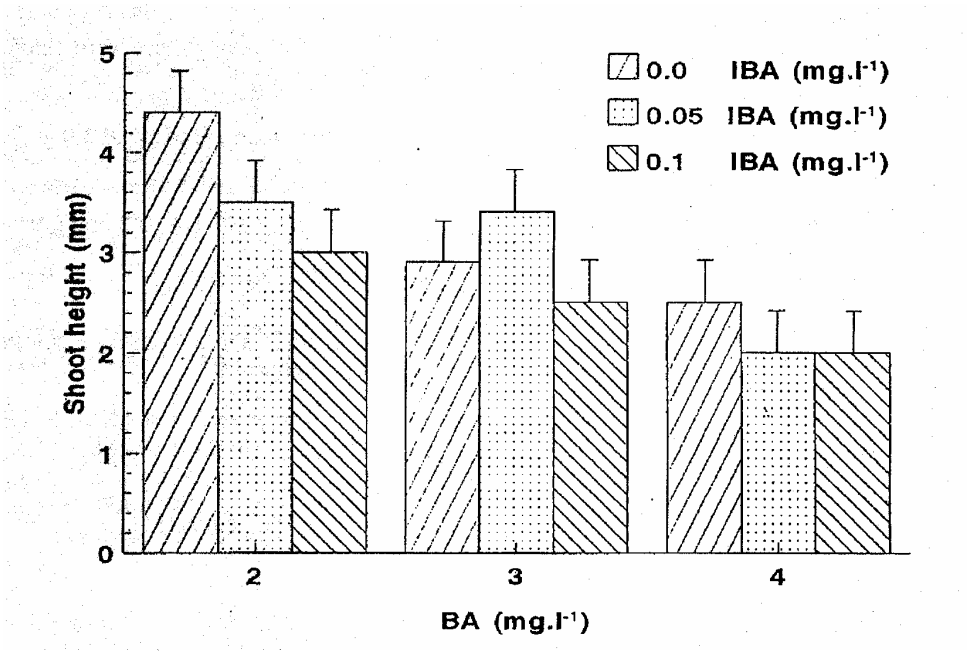


**FIGURE 5. Effect of BA and IBA concentration on shoots height growth of black wattle at 40$^{th}$ day. Vertical lines represent Cl (p = 0.05).**

**Shoots elongation**

The reduction in the levels of BA in the basal medium allowed the shoots elongation. Up to 4 elonged shoots per explant were obtained with height varying from 10 to 20 mm long at 30 days of culture. A higher uniformity in the shoots height was observed in the presence of BA (0.5 mg.l$^{-1}$). BA reduction to 0.05 mg.l$^{-1}$ allowed a non uniform growth of the shoots. A higher number of explants were obtained in this treatment, with at least one shoot with 20 mm long.

The absence of BA inhibited the shoots growth. A higher frequency of shoots with apical necrosis was observed in this treatment at 30 days of culture. BA seemed to be suitable for the maintenance of the shoots vigor. It may probably have occurred a synergistic effect between BA and culture medium. The same did not occurre between the medium nutritional balance and plant. It would be important the definition of a specific medium for the species and/or culture phase, with suitable nutritional levels to allow the shoot growth and development. Studies of this nature have been done by GONÇALVES (1980) and CORREIA (1993) for **Eucalyptus urophylla** and **Eucalyptus grandis**, respectively.

**Shoots rooting**

The formation of callus at the explant base with no root induction was observed at the 5$^{th}$ day of culture. Some explants presented yellow color in the leaves and/or falling leaves at 20 days of culture.

The individualization of the shoots was necessary for the in vitro rooting phase, providing a better seedling quality. However, this process could cause the lost of shoots vigor resulting from a possible dependence to the explant itself (shoot clusters). In addition, connected studies for the definition of the ideal auxin level, frequency of subcultures in the following phases and nutritional questions on the medium could help in the obtention of suitable answers to the shoots rooting of black wattle.

## CONCLUSIONS

- the shoots isolation and induction with growth up to 10 mm long at 7 days of culture were obtained in MS half strength with supplement of BA and IBA at 0.05 $mg.l^{-1}$, respectively;
- the shoots multiplication may be obtained in MS half strength with supplement of BA at 3.0 $mg.l^{-1}$ and IBA at 0.05 $mg.l^{-1}$;
- elonged shoots up to 20 mm long may be obtained in MS half strength with BA (0.5 and 0.05 $mg.l^{-1}$);
- there was no shoot rooting in MS half strength with supplement of IBA at 1.0 $mg.l^{-1}$;
- studies of medium specialty for the species x subculture frequency and periodicity x cultures growth conditions may allow the advance of micropropagation for black wattle.

## ACKNOWLEDGEMENTS

The authors acknowledge T ANAGRO S.A for the partial funding of this research.

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Trabalho recebido = 14/07/1994
Trabalho aceito = 23/06/1995