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Plant Regeneration of *Ilex paraguariensis* (*Aquifoliaceae*) by *in vitro* Culture of Nodal Segments

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ABSTRACT: *In vitro* regeneration of complete plants from nodal single bud segments of "yerba mate" (*Ilex paraguariensis* St. Hil.) was studied under defined nutritional and environmental conditions. Nodal segments harvested from actively growing shoots of conventionally raised plants were cultured on nutrient medium with the mineral salts and vitamins of Murashige and Skoog medium at 1/4 strength, supplemented with various concentrations of sucrose and 6-benzyladenine (BAP).

Shoot regeneration from explants of both young (2 years old) and adult (20 years old) mother plants were readily achieved in the medium supplemented with 0.04 – 0.09 M sucrose with or without BAP. As many as 60 – 65% of the nodal segments cultured formed shoots. Rooting of regenerated shoots was observed in 50% of the explants harvested from young plants, whereas 25% of the explants rooted when the nodal explants were harvested from adult plants. The best rooting induction was achieved on 1/4 strength MS medium with vermiculite as the substrate and supplemented with 1 – 1.5% IBA (indolebutyric acid) and 1 – 2% PPZ (3- methyl – 1 – phenyl – 2 pyrazolin – 5 – one). Plantlets were successfully transferred to soil.

Introduction

The "mate tree" (*Ilex paraguariensis*, *Aquifoliaceae*) – a perennial crop – is an important source of income of some regions of South America (Northeast of Argentina, Paraguay and South of Brazil). Its leaves are used for making a stimulatory beverage named "mate". Because of its allogamy, asexual reproduction would be of great value for the multiplication of selected clones. Successful micropropagation is possible for juvenile material (Sansberro *et al.*, unpublished). By culture of nodal segments of adult material (more than 1 year old), shoot regeneration can be readily achieved, however successful establishment of explants depends on proper explant selection (Bernasconi *et al.*, 1996; Mroginski *et al.*, 1996) as well as the medium composition (Rey *et al.*, 1991). However, plant regeneration of explants from mature tissue is limited mainly because of the difficulties encountered in inducing roots from regenerated shoots (Mroginski *et al.*, 1997).

This study was designed to optimize the culture medium for *in vitro* shoot production and elongation of cultures derived from both, young and adult "mate trees". This report provides information on the effect of sucrose level as the carbon source. Also, rooting and soil establishment of regenerated shoots are reported in this paper.

Material and Methods

Plant material and disinfection

The explants of *Ilex paraguariensis* St. Hil. (clone G18) used for *in vitro* culture were 0.5 to 1.0 cm long segments of stems bearing a node and a portion of underlying internode. These explants were referred to as nodal segments. Explants were taken from nonlignified branches of two lots of mother plants (Fig1):

A) Plants (2 years old) produced from seed.

B) Plants obtained by stakes of field grown adult plants (20 years old).

Both lots of mother plants were maintained in greenhouse conditions.

Nodal segments were surface sterilized by soaking for 2 min in 70% ethanol followed by immersion in a solution of commercial bleach (containing 1.2% sodium hypochlorite, final concentration) for 20 minutes. The explants were then rinsed three times in sterile distilled water.

Culture medium

The culture medium was that reported by Rey *et al.* (1991) for *Ilex paraguariensis*. It consisted of quarter-strength Murashige and Skoog (1962) medium (¼MS) containing 0.02 to 0.2 M sucrose, 0.6 % Sigma agar (A-1296) and supplemented with or without 0.1 mg/L benzylaminopurine (BAP). The pH of each medium was adjusted to 5.8 with KOH or HCl prior the addition of agar. Tubes were covered with aluminium foil and autoclaved at 1.46 kg/cm^2 for 20 minutes.

Physical culture conditions

The nodal segments were cultured on 10 cc synthetic medium in a 40 cc glass tubes. The tubes were sealed with Resinite AF 50® (Casco S.A.C. Company) and incubated in a growth room. The temperature was maintained at 27 ± 2°C with lighting (cool white fluorescent light) at 116 µmol m^{-2} s^{-1} and 14 hr photoperiod.

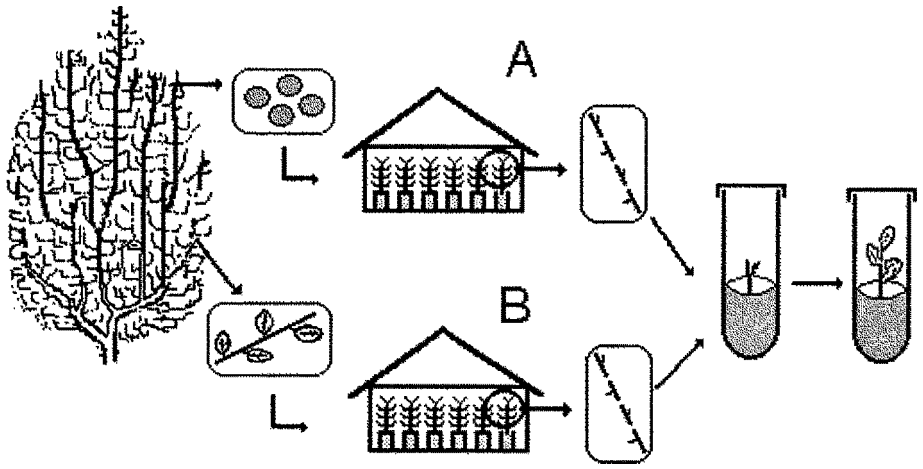

FIGURE 1. Source of explants for plant regeneration from nodal segments of *Ilex paraguariensis*. A. seedlings 2 years old B. Plants obtained by stakes of adult plants (20 years old).

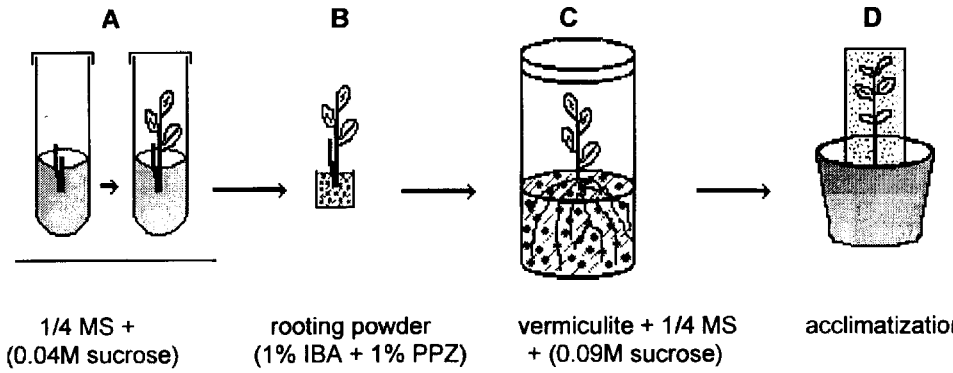

FIGURE 2. Plant regeneration technique from nodal segments of *Ilex paraguariensis* A- Shoot regeneration, B and C rooting of regenerated shoot, D plantlet transferred to soil.

Chemical analysis

For quantification of total chlorophyll the AOAC methods 6098, 6099, 6100 were employed (Horwitz, 1960). For quantification of anthocyanins, they were extracted overnight from fresh cells with ethanol and 1% HCl (85:15). Optical density of the extracts was measured at 535 nm. The anthocyanin concentration was calculated using the extintion coefficient ($E^{1\%}$= 98.2 at 535 nm) for cranberry anthocyanin extracted in the same solvent (Francis, 1982).

Results analysis

Each treatment consisted of 9 – 10 explants and each experiment was repeated at least 3 times. Means are given with the standard error (± SE).

Rooting of regenerated shoots

Shoots of 1 - 2 cm in length with the primary explant were used for root initiation. These shoots were regenerated after 45 days of culture of nodal segments on ˇ MS medium with 0.04M sucrose (Fig.2A).

The rooting technique (Fig. 2) consisted of applying the rooting powder (1% IBA + 1% PPZ) to the base of the explants containing the regenerated shoots (Fig. 2B) and subsequently transfering them to ˇ MS medium with 0.09M sucrose, with or without 1 or 2% PPZ (3-methyl-1-phenyl-2-pyrazolin-5-one) and 1.0 or 1.5% indolebutyric acid (IBA). The support of the shoots was vermiculite grade 2 contained in 170 cc jars (Fig.2C).

For the rooting experiments, 10 shoots were used *per* treatment and each experiment was repeated 3 times. Rooting was evaluated 120 days after the experiment began.

The physical conditions for rooting experiments were the same as described above for nodal segment cultures.

The rooted plants were transplanted in pots containing a mix of sand and soil (1:1) and covered with plastic bags for 3 weeks to prevent desiccation and to allow acclimatization.

Results and Discussion

After 5-10 days of culture in most of the media tested in this work, the three typical growth responses were evident: 1) browning of the explants, 2) contamination with microorganisms (fungi and/or bacteria) and, 3) uncontaminated nodal segments which remain green. The 3rd set of explants were considered in this work as established. These results are in agreement with our previous works (Rey and Mroginski, 1988; Rey *et al.*, 1991; Bernasconi *et al.*, 1996; Mroginski *et al.*, 1996). The effect of five concentrations of sucrose on the percent of nodal segments established from both, young and adult plants is shown in Fig. 3. It can be observed that lowest levels of sucrose (0.02 to 0.09 M) were more favorable to establish the explants. On the contrary, the highest concentrations of sucrose (0.15 and 0.20 M) resulted in a decrease in the number of nodal segments successfully established with the subsequent increase in the browning of the explants. This response has been also reported for other woody plants and could be explained as the result of the increase of the osmotic pressure of the medium (Thompson and Thorpe, 1987). In addition, by using low sucrose concentration (0.04 M) it was possible to successfully establish explants of *Ilex paraguariensis* along different seasons of the year (Sansberro, unpublished) which was earlier found to be as impossible in winter months (Bernasconi *et al.*, 1998).

Nodal segments of young plants showed maximum frequency of shoot regeneration (60%) when the medium contained 0.09 M sucrose, whereas it was 65% for adult explants cultured with 0.04 M sucrose. (Fig. 4). After four weeks culture, shoots regenerated on nodal segments were 0.5-1.0 cm long. Thus, in the case of shoot regenerated from young plants, the maximum growth occurs at 0.04 or 0.09 M sucrose, and growth decreased at 0.02 M sucrose as well as at 0.15 M sucrose, while growth of the shoots at 0.20 M sucrose was insignificant (Fig. 5). It is interesting to note that roots differentiated in some explants cultured at 0.15 M sucrose (Fig. 5). With shoots regenerated from adult plants, the maximum growth occurs at 0.04 M sucrose (Fig. 6). In all cases, 1 shoot/ explant could be excised for subculture. The shoots remained unbranched. The importance of sucrose for micropropagation has been stressed by many workers (Jusaitis, 1997; Marino *et al.*, 1993). In addition, because a reduction of carbohydrate content generally increases photosynthetic rates (Langford and Wainwright, 1987; Galzy and Compan, 1988; Hdider and Desjardins, 1994) and promotes autotrophy (Deng and Donnelly, 1993), reducing sucrose in the medium might have a positive influence on acclimatization.

When young and adults explants were maintained for 6 weeks in medium with high levels of sucrose, it had been possible to observe a significant reduction in the size of the regenerated shoot (Fig. 7). In addition, it

had been also possible to appreciate changes in the colour (from green to red) of the original explants as well as in the resulting shoots and leaves. Similar results are reported for *Eucalyptus sideroxylon* (Cheng *et al.*, 1992). This response would seem to result not only from the increased of anthocyanin production but from lower chlorophyll synthesis when the media have high concentration of sucrose (Table 1). These results differ from those obtained in other plant species such as, *Oxalis linearis* (Meyer and Van Staden, 1995), and strawberry (Sato *et al.*, 1996), where the change in color of the explants from green to dark-red with increasing sucrose levels may be due to an increase of anthocyanin biosynthesis.

The experiments conducted to improve the regeneration performance of nodal segments by using different concentrations of sucrose with 0.1 mg/L BAP did not show a favorable response (Fig. 8). In terms of percentage of explants which produced shoots -even in the case of nodal segments of adult plants- the percentage was significantly lower than that cultured without BAP. However, the addition of BAP provoked the production of multiple shoots (in average 3-5 shoots *per* explant cultured (Fig. 9). These results are in agreement with

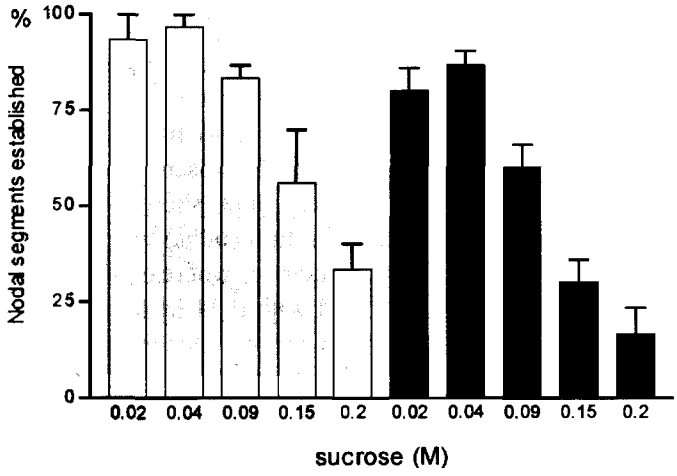

FIGURE 3. Effect of sucrose concentrations in 1/4 MS medium on the *in vitro* establishment of nodal segments of young (❑) or adult (■) plants of *Ilex paraguariensis*. Results after 10 days culture. Vertical bars indicate ± SE.

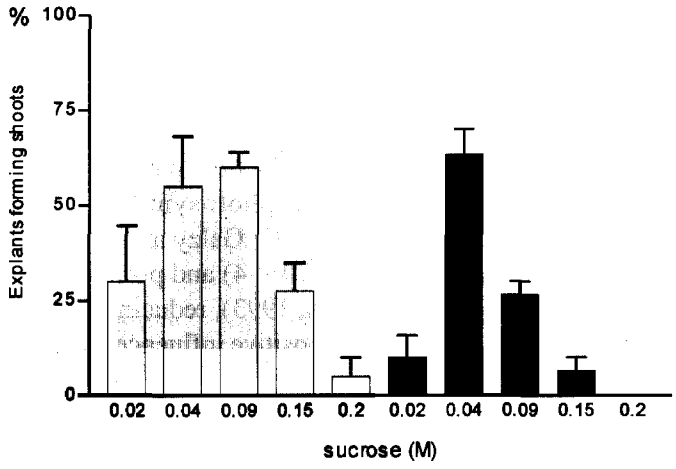

FIGURE 4. Effect of different concentrations of sucrose in 1/4 MS on the shoot production by culture of nodal segments of young (❑) or adult (■) plants of *Ilex paraguariensis*. Results after 45 days culture only shoots more than 0.5 cm in length were scored. Vertical bars indicate ± SE).

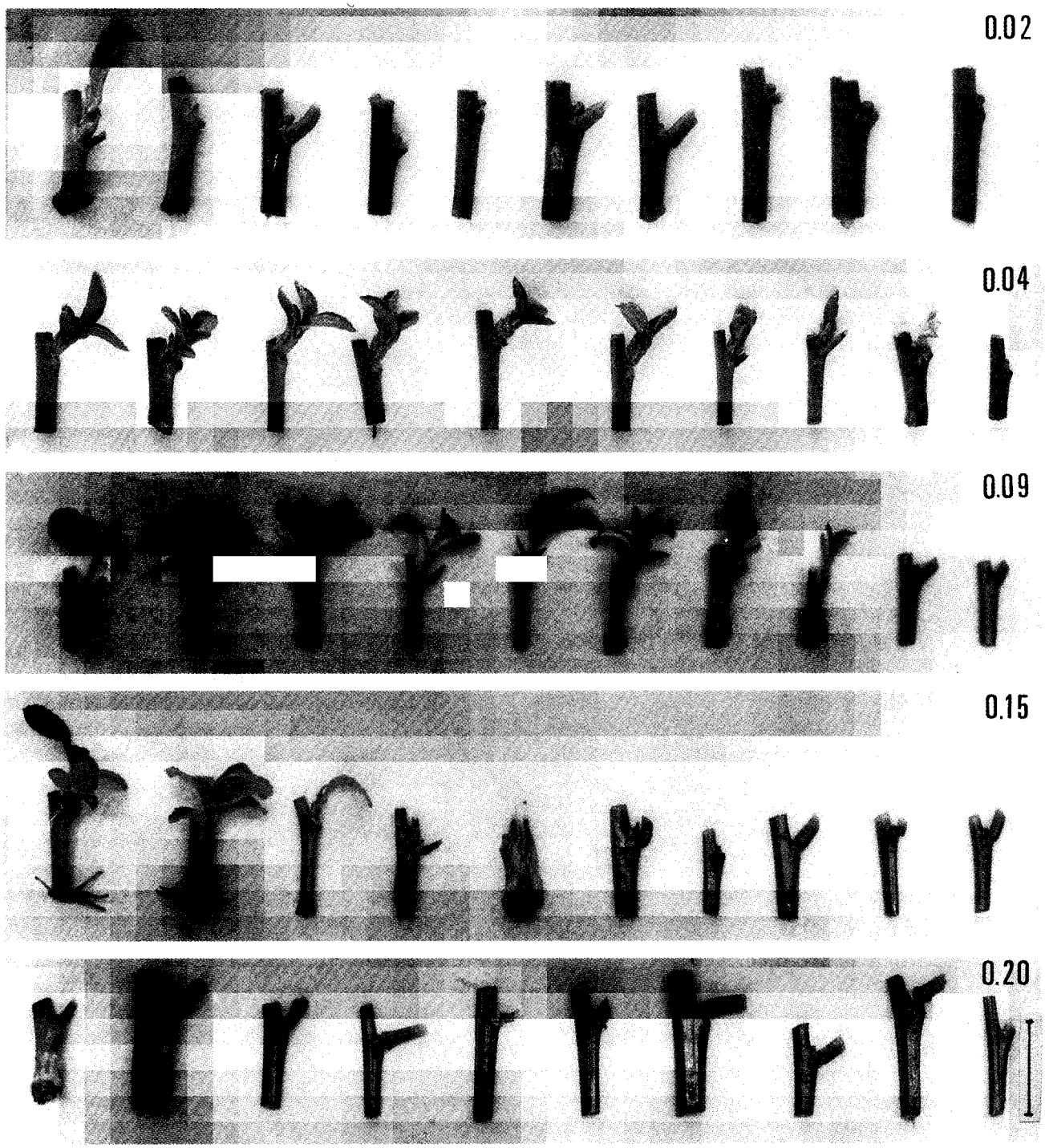

FIGURE 5. Effect of the sucrose concentrations (in M) on the shoot regeneration from nodal segments of young mother plants of *Ilex paraguariensis*. Bars represent 1 cm.

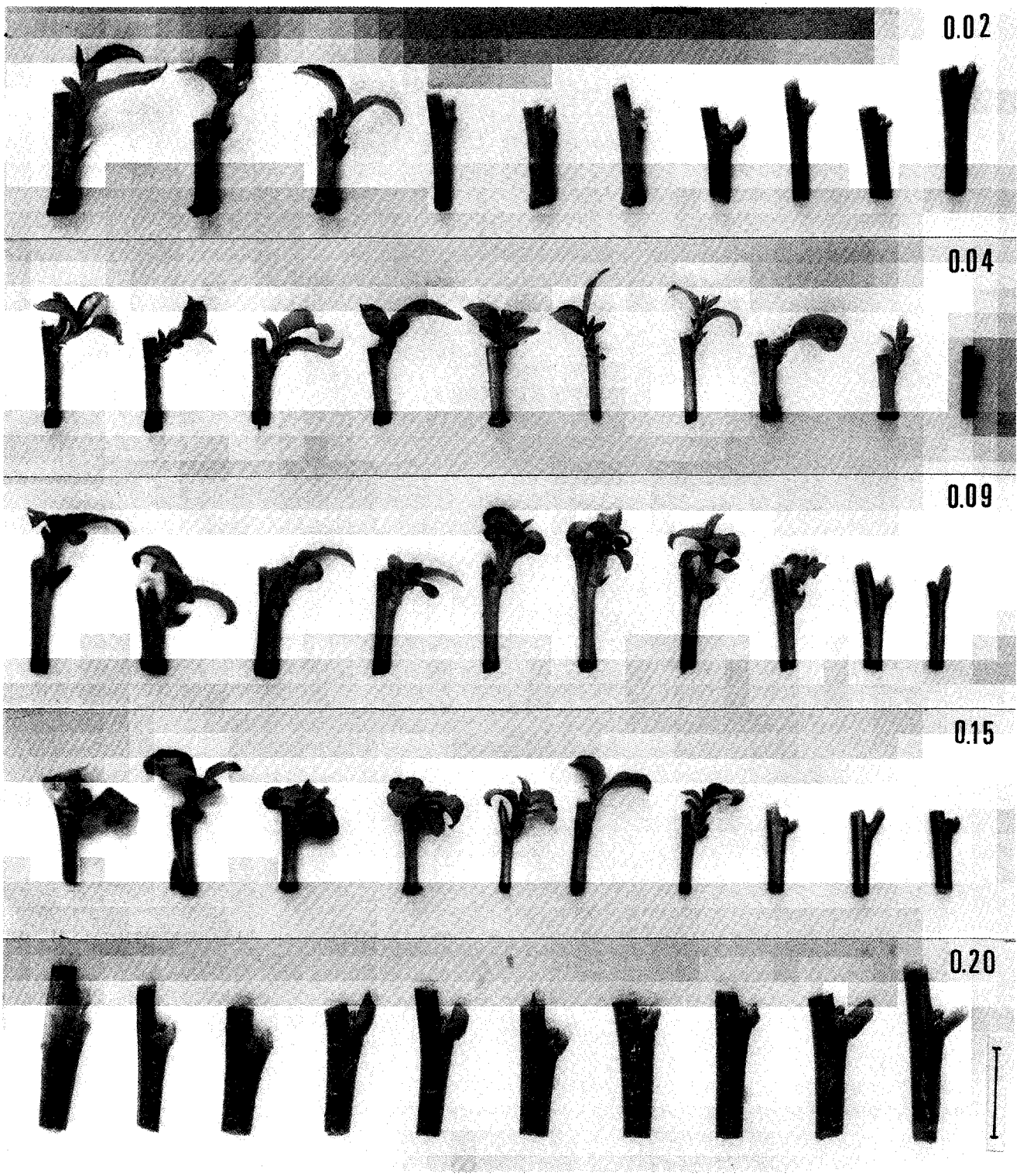

FIGURE 6. Effect of the sucrose concentration (in M) on the shoot regeneration from nodal segments of adult mother plants of *Ilex paraguariensis*. Bars represent 1 cm.

TABLE 1.

Effect of five concentrations of sucrose on the production of chlorophyll and anthocyanins by shoot regenerated by in vitro culture of nodal segments of Ilex paraguariensis in ˇ MS.

Sucrose (M)	Total chlorophyll (%)	Anthocyanins (ppm)
0.02	1.30 ± 0.09	164.0 ± 11.50
0.04	1.60 ± 0.07	203.7 ± 12.09
0.09	0.85 ± 0.09	268.7 ± 8.70
0.15	0.60 ± 0.03	188.7 ± 18.61
0.20	0.29 ± 0.07	155.7 ± 16.80

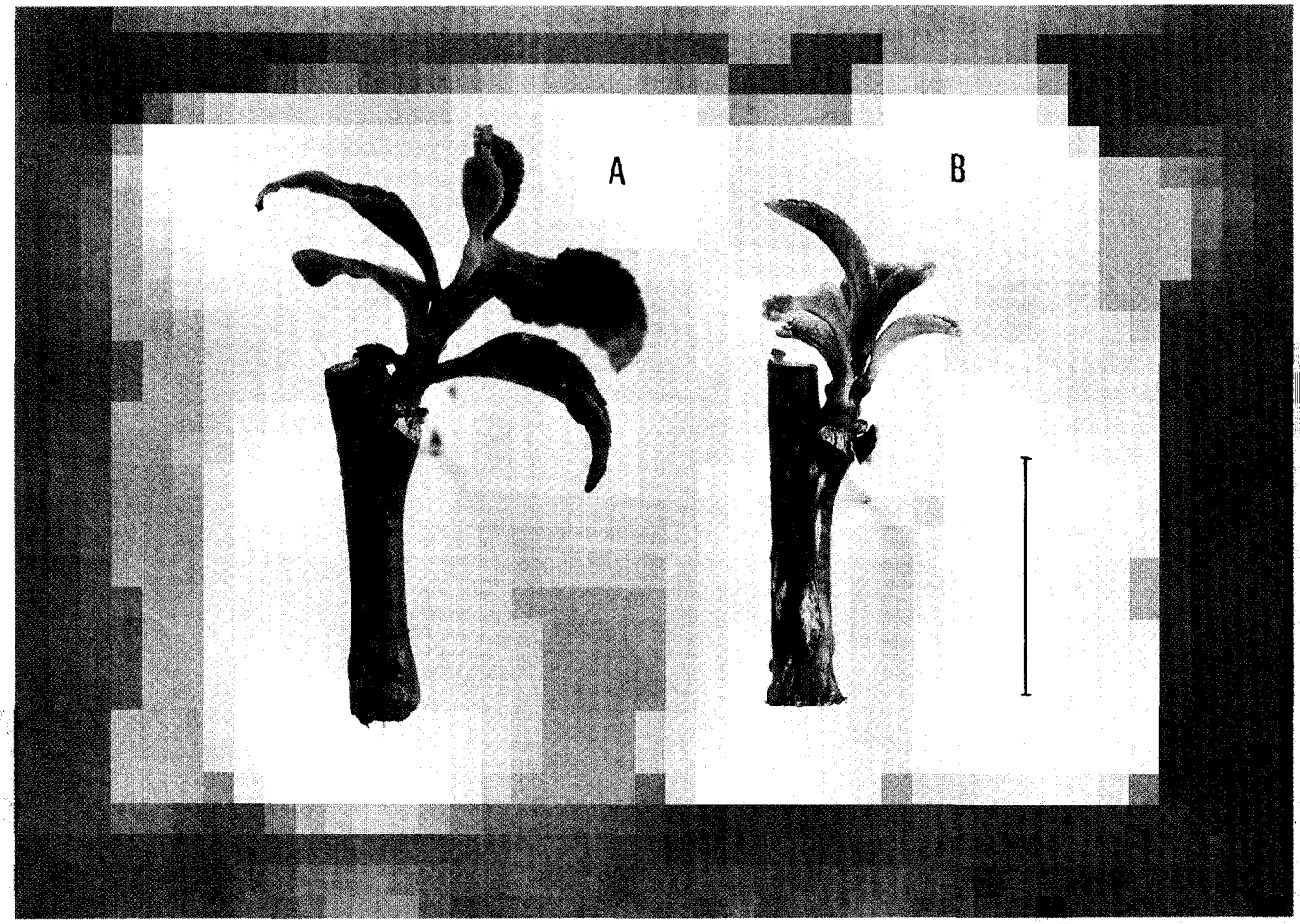

FIGURE 7. Shoot regeneration from nodal segments of *Ilex paraguariensis* cultured on 1/4 MS + 0.09 M sucrose (A) or on 1/4 MS + 0.20 M sucrose (B). Bars represent 1 cm.

our previous findings in *Ilex paraguariensis* (Mroginski *et al.*, 1999).

Rooting of regenerated shoots was readily achieved by culture the shoots according to the protocol of the Fig. 2. The weakest step in the micropropagation of mature *Ilex paraguariensis* material has been the rooting stage (Mroginski *et al.*, 1997). Most of the studies have used an agar medium for *in vitro* root development (Bernasconi *et al.*, 1996; Rey and Mroginski, 1988; Mroginski *et al.*, 1996). In the present study we found the beneficial effect of the use of vermiculite as rooting substrate (Fig. 10A) in a sterile jar. These results are in agreement with those obtained with chesnut, where the use of a peat: perlite: vermiculite (1:1:1) substrate was found to be very useful (Sánchez *et al.*, 1997).

In most treatments, shoots readily initiated roots

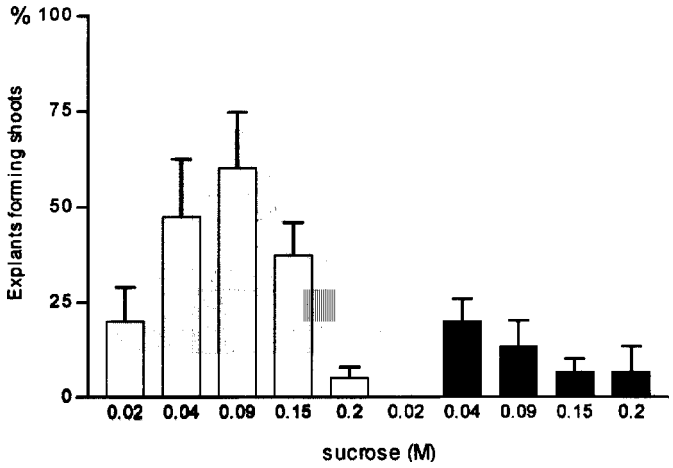

FIGURE 8. Effect of sucrose concentrations in 1/4 MS medium supplemented with 0.1 mg/L BAP on the shoot production by culture of nodal segments of young (❑) or adult (■) plants of *Ilex paraguariensis*. Results after 45 days culture. Only shoots more than 0.5 cm in length were scored. Vertical bars indicate ± SE.

FIGURE 9. Multiple shoot regeneration from nodal segments of *Ilex paraguariensis* cultured on 1/4 MS medium + 0.09M sucrose + 0.1 mg/L BAP. Bar represent 1 cm.

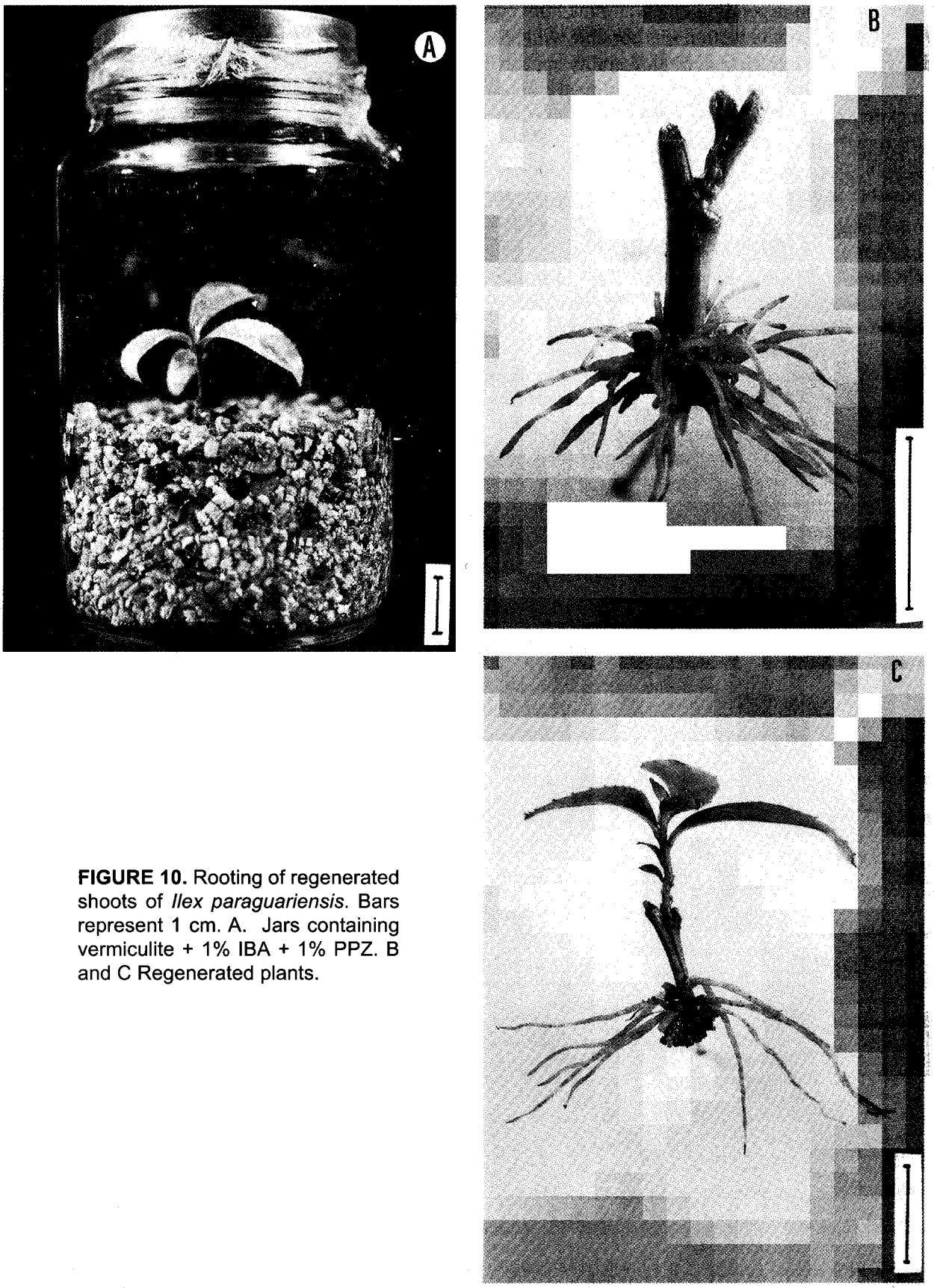

FIGURE 10. Rooting of regenerated shoots of *Ilex paraguariensis*. Bars represent 1 cm. A. Jars containing vermiculite + 1% IBA + 1% PPZ. B and C Regenerated plants.

within 3 weeks, but rooting was preceded by a small callus proliferation depending on culture medium employed. Although root induction was possible with both kind of explants (Fig. 10B,C), rooting ability appeared to be strongly related to the age of the original explant. In our experiments, there was a noticeable difference in *in vitro* rootability between young and adult explants. While with young material (2 years old), root induction was possible in all the treatments allowing the rooting of 20 – 50% of the explants (Fig. 11A), with adult material (20 years old) the rooting rate dramatically diminished to 5 – 20% of the cultured nodal segments and it was only possible in some culture media (Fig. 11C). Likewise, the number of roots differentiated was greatly affected by the age of the original explants. While with young material an average of 5 – 10 roots/ explant were scored (Fig. 11B), with adult material, in average no more than 5 roots/explant were scored (Fig. 11D). Differential responses to *in vitro* rooting ability of young and adult explants have been reported with many woody species (Capuana and Giannini, 1997; Sánchez *et al.*, 1997; Monteuuis, 1987).

Although all culture media allowed root formation from young nodal segments, some combination of IBA

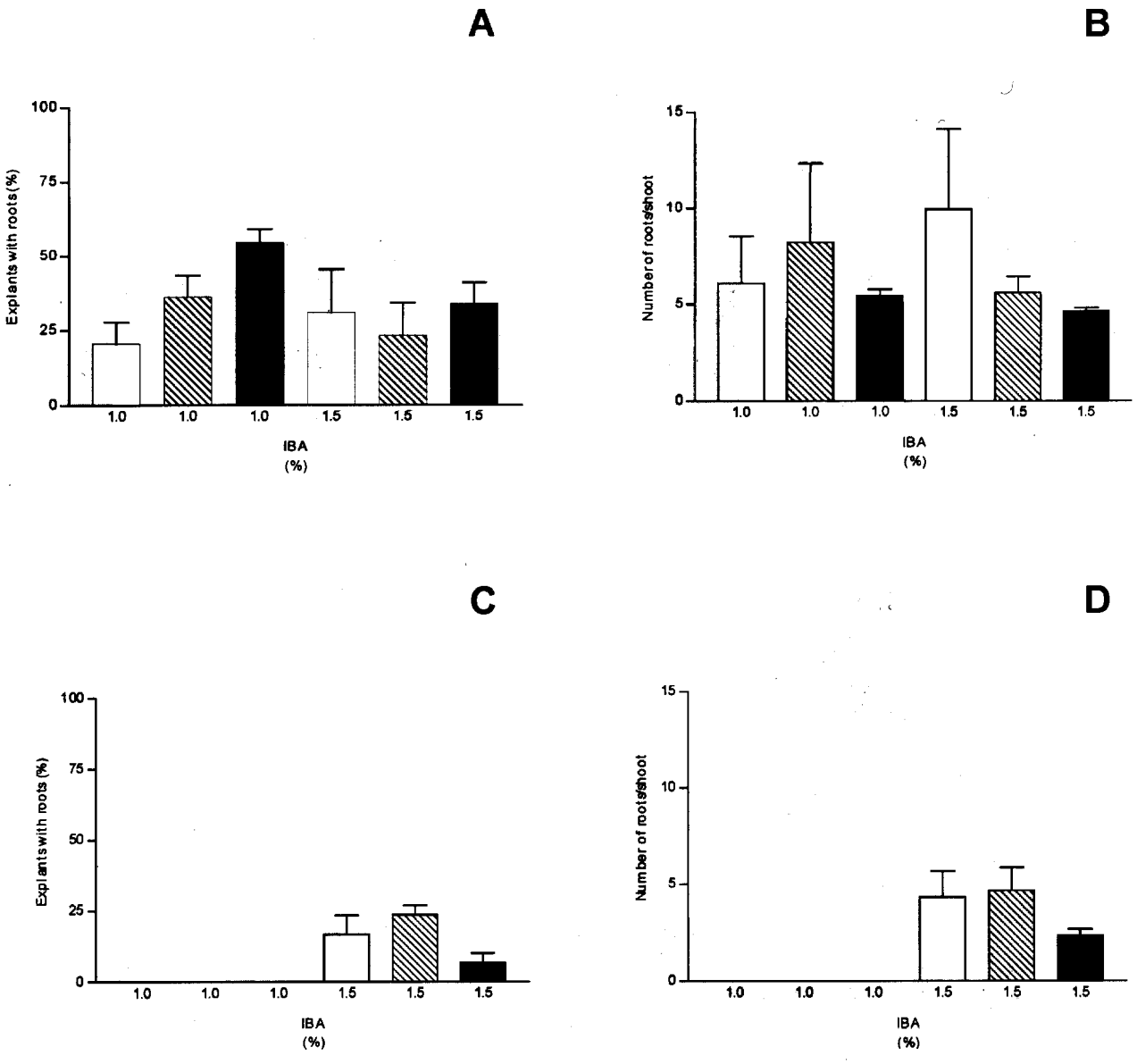

FIGURE 11. Effect of IBA and with 0 (❑), 1% (▨) or (■) 2% PPZ on the rooting of regenerated shoots of young (A and B) and adults (C and D plants).

and PPZ enhanced rooting ability (Fig. 11A). The highest percentage of shoots forming roots was observed in the medium containing 1% IBA and 2% PPZ. However, when adult explants were cultured, the best combination included 1.5% IBA and 1% PPZ. These results are in agreement with those reported by Caso and Dotta (1997) who, working with cutting from stem segments of greenhouse- grown plants of *Ilex paraguariensis*, founded that there is a synergistic action on rhizogenesis between IBA and PPZ.

The regenerated plantlets were successfully transplanted to soil. A 60% survival rate was obtained when rooted plants were planted in the greenhouse. All of them exhibited a normal phenotype, at least at early stages of growth.

In summary, this work demonstrates that complete *Ilex paraguariensis* plant regeneration from both young and adult mother plants could be performed by *in vitro* culture of nodal segments.

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