

IN VITRO MULTIPLICATION OF *Swietenia macrophylla* KING (MELIACEAE) FROM JUVENILE SHOOTS**MULTIPLICAÇÃO IN VITRO DE *Swietenia macrophylla* KING (MELIACEAE) A PARTIR DE MATERIAL JUVENIL**Eliane de Souza Schottz¹ Antonio Nascim Kalil Filho² Anderson Luiz Tracz³ Henrique Koehler⁴
Luciana L. F. Ribas⁵ Marguerite Quoirin⁶**ABSTRACT**

Big-leaf mahogany (*Swietenia macrophylla* King) is an important species for timber production that is considered the most valuable in the world. For this reason its exploitation is indiscriminate and leads this species to the risk of extinction. Moreover, mahogany is difficult to regenerate naturally and, when used in reforestation programs, plants are severely damaged by the shoot-borer (*Hypsipyla grandella* Zellar). This work aimed at developing the multiplication stage of micropropagation of *Swietenia macrophylla* King using juvenile material. After desinfestation, seeds were germinated in MS solid culture medium. Shoot formation from seeds occurred during five months, giving 5.54 nodal segments per seed. These explants were excised, each containing one axillary bud, and transferred on multiplication media. Four experiments with cytokinins were conducted, using media supplemented with 6-benzylaminopurine (BAP) (2.5 to 50.0 μM), 2-isopentenyladenine (2-iP) (0; 1.1 to 8.8 μM), combinations of BAP (0; 2.5 to 50.0 μM) and 2-iP (2.2 μM). For the first treatments the basal culture medium was MS medium and in the last one MS and QL media were used in separate experiments. When BAP was tested alone, the maximum point of multiplication rate average was obtained on medium containing 23.61 μM , while 2-iP did not induce bud multiplication. On QL culture medium supplemented with the combinations of BAP (0; 2.5; 5.0; 10.0 e 20.0 μM) and 2-iP (2.2 μM), there was no multiplication. The maximum point of multiplication rate average was 5.7 μM , obtained when the MS culture medium was supplemented with 18.51 μM BAP and 2.2 μM 2-iP.

Keywords: Cytokinins; mahogany; micropropagation; tropical species.

RESUMO

Swietenia macrophylla King (mogno) é uma espécie arbórea nativa da Amazônia cuja madeira é considerada uma das mais nobres do mundo. Por esse motivo, vem sofrendo grande pressão de exploração, colocando-a entre as espécies em risco de extinção. Além disso, possui dificuldade de regeneração natural e de estabelecimento em reflorestamentos, sendo atacado por larvas de *Hypsipyla grandella* Zellar. Este trabalho teve como objetivo desenvolver a etapa de multiplicação *in vitro* de mogno. Após a desinfestação, as sementes foram colocadas para germinar em meio de cultura MS completo. Após 6 semanas de germinação, os caules foram cortados em segmentos nodais, cada um contendo uma gema axilar. Foram realizados quatro experimentos com meios de cultura acrescidos de 6-benzilaminopurina (BAP) (2,5; 5; 10; 20 e 50 μM), 2-isopenteniladenina (2-iP) (0; 1,1; 2,2; 4,4 e 8,8 μM) e combinações de BAP (0; 2,5; 5; 10; 20 e 50 μM) e 2-iP (2,2 μM). Nos dois primeiros tratamentos (BAP e 2-iP isoladamente), o meio de cultura MS foi utilizado como meio básico e, nos últimos tratamentos, utilizaram-se os meios MS e QL. Quando BAP foi testado isoladamente, o ponto máximo da taxa média de multiplicação foi de 23,61 μM , enquanto que não houve multiplicação na presença de 2-iP. O meio de cultura QL, suplementado com as combinações de BAP (0; 2,5; 5; 10 e 20 μM) e 2-iP (2,2 μM), não induziu a multiplicação dos brotos. O ponto máximo da

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taxa média de multiplicação foi de 18,51 μM , obtido com o uso do meio de cultura MS acrescido de BAP e 2,2 μM de 2-iP.

Palavras-chave: Citocininas; mogno; micropropagação; espécie tropical.

INTRODUCTION

Big-leaf mahogany (*Swietenia macrophylla* King), also known as "mogno", "caoba" and "mara" in tropical America, belongs to the Meliaceae family. Distributed from Mexico to the Amazonian region of Brazil, it is one of the most valuable timber trees in the world. It is used for furniture manufacturing, general joinery work, fine veneer production and boat construction (Lamb, 1996).

There are several reports of *in vitro* tissue culture of this species. Lee and Rao (1988), using stem segments from seedlings and from the basis of 10-year-old trees coppice, observed the formation of adventitious shoots from calli, when the nodal segments were cultivated on MS (Murashige and Skoog, 1962) medium supplemented with 8.87 or 22.2 μM BAP. Maruyama and Ishii (1997) reported the use of WPM (Lloyd and McCown, 1980) mineral medium supplemented with 10 μM zeatin for multiplication stage of mahogany. A patent developed by a Japanese group (Patent No JP09019229; 21.01.1997) described a mass propagation method, using apical or axillary buds to induce proliferation in liquid medium under agitation. Medium B₅ (Gamborg *et al.*, 1968), in its original composition or modified, supplemented with 0.09 to 4.44 μM BAP and/or 0.1 to 4.9 μM IBA, induced polybud formation. The regeneration of adventitious buds from epicotyl explants was reported by Valverde-Cerdas *et al.* (1998). The basal medium was constituted of half-strength MS salts and was supplemented with BAP. Venketeswaran *et al.* (1988) tested MS and WPM culture media containing kinetin (4.65 μM) or BAP (17.8; 44.85 and 89.77 μM). The work of Albarran *et al.* (1997) described the development of axillary buds from nodal segments cultured on half-strength MS mineral medium containing 8.87 μM BAP and 11.42 μM indolacetic acid (IAA) in only 10% of the explants. Lopes *et al.* (2001) reported the multiplication of the same species on MS medium supplemented with 4.44 μM BAP and 0.0054 μM naphthaleneacetic acid (NAA), but did not indicate the multiplication rate. This work describes the results of multiplication experiments in which two mineral salt formulations and two cytokinins, isolated or in combinations, were tested for micropropagation of big-leaf mahogany.

MATERIAL AND METHODS

Seed origin – *Swietenia macrophylla* seeds of open pollination were collected from 20 trees in Brasnorte, Mato Grosso (Brazil), and stored in dry room at 15°C and 20 to 25% relative humidity.

Seed desinfestation and germination – The teguments were removed manually and the desinfestation made with Benomyl at 0.1% (p/v), followed by treatment with NaOCl solution at 2% (v/v) containing Tween-20 at 0.1% (v/v). Both treatments were done in agitation, during 10 minutes. The seeds were then rinsed three times in sterile distilled water. After desinfestation, seeds were germinated on MS medium solidified with 7 g. L⁻¹ agar (Difco Bacto®). Each seed was cultured in a flask of 6 cm diameter and 13 cm high. Every plantlet and the shoots originated from it were considered as a clone.

Culture conditions – The conditions of growth chamber were: temperature of 27±2°C, cold white light (Philips®) giving a photon flux density of approximately 40 $\mu\text{mol. m}^2.\text{s}^{-1}$ and a photoperiod of 16 h.

Micropropagation – The epicotyls were excised from six-weeks-old plantlets and used to initiate culture of nodal segments, each one bearing one axillary bud. After excision of epicotyls, the plantlets were maintained *in vitro* to allow the growth of secondary shoots from the cotyledonary node. For the culture of secondary shoots, every new shoot was cut in nodal segments that were cultured in flasks of 6.5 cm diameter and 8 cm high, containing 40 ml of medium. During five months, new shoots were cut from the mother plant and divided into nodal segments. In the experiments with cytokinins, three segments were cultured per flask of 6.5 cm diameter and 8.5 cm high, containing 40 ml of medium. The experimental unit was composed of ten flasks. All media contained 3% sucrose and 7 g. L⁻¹ agar (Difco Bacto®). After adjusting pH to 5.8, they were autoclaved at 120° for 20 min. Two cytokinins were tested in separated experiments: BAP (2.5; 5.0;

10.0; 20.0 and 50.0 μM), 2-iP (0; 1.1; 2.2; 4.4 and 8.8 μM) and a combination of both (2.2 μM 2-iP with 0; 2.5; 5.0; 10.0; 20.0 and 50.0 μM BAP). All these treatments were added to MS medium. In addition, the combinations of both cytokinins (2.2 μM 2-iP with 0; 2.5; 5.0; 10.0 and 20.0 μM BAP) were added to QL (Quoirin and Lepoivre, 1977) mineral media combined with MS vitamins. After 45 days the number of new buds or shoots, of buds per new shoot and the shoot size were recorded. The shoots were defined as organs formed by a stem with leaves and axillary buds. Multiplication rate was evaluated dividing the number of buds or shoots obtained after 45 days by the initial number of nodal segments.

Statistical analysis – All experiments were completely randomized. The number of explants per flask was three. The number of replicates was 12 in the experiment with BAP, 36 with 2-iP, and 8 with combinations of BAP and 2-iP. Data were analyzed by factorial ANOVA was performed on data using MSTATC (Michigan State University). Significance was recorded at $P < 0.05$. For all experiments, regression equations were adjusted to show the relationship between the concentrations tested and the independent variables evaluated.

RESULTS AND DISCUSSION

The formation of secondary shoots from the cotyledonary nodes after every excision occurred during five months. The number of nodal segments varied in function of the clone, giving a total of 133 segments from 24 clones and a mean rate of 5.54 segments per clone. In the experiments testing the effect of cytokinins on axillary bud proliferation, the BAP concentration of 23.61 μM was the most efficient to induce bud multiplication on MS culture medium and concentrations of 50.0 μM inhibited bud formation, confirming the results of Figure 1, after 45 days. The correlation between BAP concentrations and multiplication rate was 0.92, being represented by a second degree polynomial equation ($R^2 = 0.85$) (Figure 1).

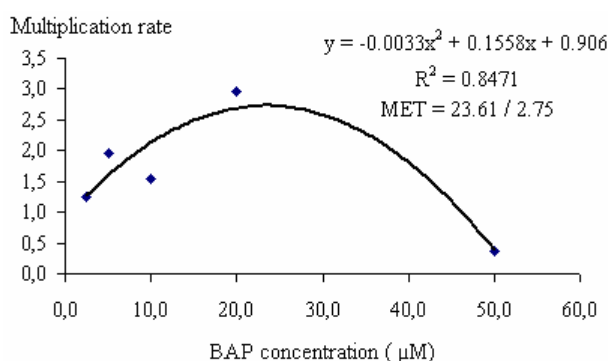


FIGURE 1: Multiplication rate of air parts of *Swietenia macrophylla*, 45 days after inoculation in function of BAP concentrations.

FIGURA 1: Taxa de multiplicação de partes aéreas de *Swietenia macrophylla*, 45 dias após a inoculação em função das concentrações de BAP.

Venketeswaran *et al.* (1988) compared kinetin to BAP for mahogany micropropagation and observed a better shoot production with BAP (17.8 μM alone or 89.77 μM with activated charcoal). Maruyama *et al.* (1989a) reported the production of multiple shoots when using BTM medium (“Broad Leaved Tree Medium”, CHALUPA, 1983) supplemented with 8.88 μM BAP. In preliminary experiments of this work, BTM medium was combined with 4.44 μM BAP, but no multiplication of buds was observed (results not shown). BTM culture medium was also supplemented with 4.56, 11.4 and 22.8 μM zeatin and some formation of buds occurred in the presence of 4.56 μM (data not shown), differently from the results reported by Maruyama and Ishii (1997) who observed a multiplication rate of five to seven when shoot tips were cultured on WPM mineral medium supplemented with 10 μM zeatin.

The BAP concentration of 50 μM caused inhibition of bud formation and reduced multiplication rate to 0.36; 67% of the explants were necrotic and all of them presented leaf chlorosis. The inhibition of shoot formation in the presence of high BAP concentrations was also observed by Maruyama *et al.* (1989b) in cultures of apical segments of *Cedrela odorata* on WPM medium, by Ashok and Craig (1996) for apical

segments of *Camptotheca acuminata* shoots on B₅ culture medium and by Qi-Guang *et al.* (1986) in tissue culture of *Castanea molissima*. Furthermore, it is well known that BAP in high concentrations causes hyperhydricity in the explants, interacting with the agar of culture medium. Excess of cytokinin can lead to other negative effects like the formation of a great number of adventitious buds, which is not desirable for clonal integrity (Grattapaglia and Machado, 1998).

The correlation between the 2-iP concentrations and multiplication rate was 0.7, being represented by a third degree polynomial equation ($R^2 = 0.49$). Though there was no significant difference among the results, the highest rate was observed in the presence of 2.6 μM 2-iP, according to the adjusted equation (Figure 2).

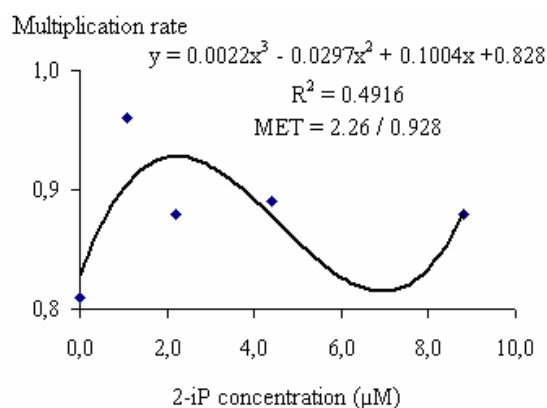


FIGURE 2: Multiplication rate of aerial parts of *Swietenia macrophylla*, 45 days after inoculation, in function of 2-iP concentrations.

FIGURA 2: Taxa de multiplicação de partes aéreas de *Swietenia macrophylla*, 45 dias após a inoculação, em função das concentrações de 2-iP.

The correlation between 2-iP concentration and bud mean number was 0.55, represented by a third degree polynomial equation ($R^2 = 0.30$) (Figure 3). George (1993) indicated that, in the presence of 2-iP, *Gerbera* explants did not multiply, due to the rapid degradation of this cytokinin by the action of cytokinin oxidase naturally present in the tissues. This fact could explain the low multiplication rates attained in this experiment.

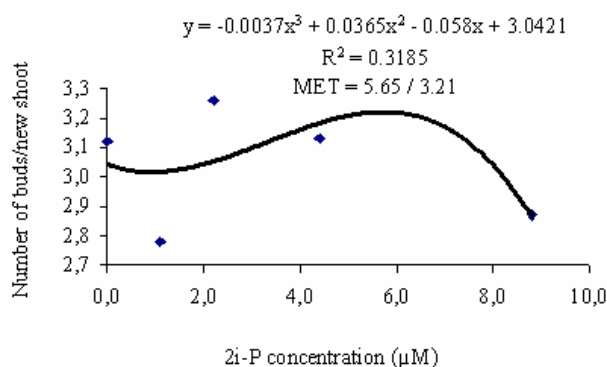


FIGURE 3: Number of buds/new shoot of *Swietenia macrophylla*, 45 days after inoculation in function of 2-iP concentrations.

FIGURA 3: Número de gemas/broto de *Swietenia macrophylla*, 45 após a inoculação, em função de concentrações de 2-iP.

Concerning mean shoot length, around 38% of shoots were shorter than 0.5 cm, 52% between 0.5 and 1 cm and only 10.5% higher than 1 cm (Figures 4 and 5). The highest percentages of shoots superior to 1 cm were obtained in the presence of 1.1 and 4.4 μM 2-iP but were only 14

and 12%, respectively. In tissue culture of *Cercis canadensis* var. *mexicana*, 2-iP did not induce formation of multiple shoots in apical segments cultivated on WPM culture medium and the highest elongation was 1.17 cm for a 2-iP concentration of 24.6 μM (Mackay *et al.*, 1995).

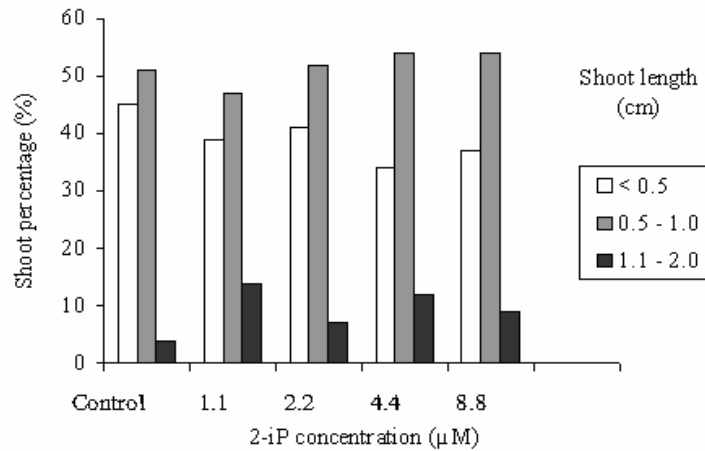


FIGURE 4: Mean percentage of shoots of *Swietenia macrophylla* with length < 0.5 cm; between 0.5 and 1.0 cm and between 1.1 and 2.0 cm in function of 2-iP concentration in MS medium.

FIGURA 4: Porcentagem média de brotos de *Swietenia macrophylla* com comprimento inferior a 0.5 cm; de 0.5 a 1.0 cm e de 1.1 a 2.0 cm em função da concentração 2iP no meio de cultura MS.

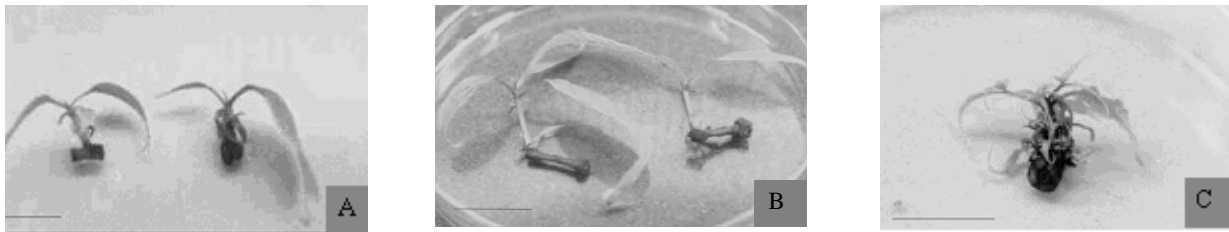


FIGURE 5: *Swietenia macrophylla* shoots after 45 days. (A) In MS culture medium without growth regulators. (B) In MS culture medium supplemented with 4.4 μM 2-iP. (C) In MS culture medium with 10.0 μM BAP and 2.2 μM 2-iP. Barra: 1 cm.

FIGURA 5: Brotos de *Swietenia macrophylla* após 45 dias. (A) Em meio de cultura MS sem adição de reguladores de crescimento. (B) Em meio de cultura MS suplementado com 4.4 μM de 2-iP. (C) Em meio de cultura MS com 10.0 μM de BAP e 2.2 μM de 2-iP. Barra: 1 cm.

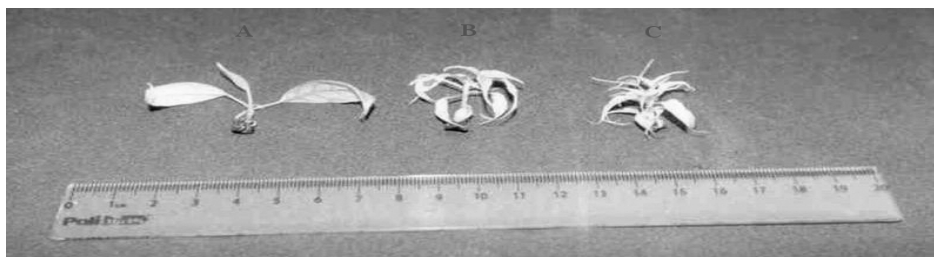


FIGURE 6: *Swietenia macrophylla* shoots after 45 days. (A) In QL culture medium without growth regulators. (B) In QL culture medium supplemented with 2.5 μM of BAP and 2.2 μM 2-iP. (C) In QL culture medium with 5.0 μM of BAP and 2.2 μM 2-iP.

FIGURA 6: Brotos de *Swietenia macrophylla* após 45 dias. (A) Em meio de cultura QL sem reguladores de crescimento. (B) Em meio de cultura QL suplementado com 2.5 μM de BAP e 2.2 μM de 2-iP. (C) Em meio de cultura QL suplementado com 5.0 μM de BAP e 2.2 μM de 2-iP.

When BAP was combined with 2.2 μM 2-iP, the maximum point of multiplication rate average was obtained on medium containing 18.51 μM BAP (Figure 7).

The correlation between BAP concentration and bud number was 0.7, represented by a third degree

polynomial regression curve ($R^2 = 0.5$) (Figure 7). The buds did not elongate into shoots. The same kind of result was obtained by Anderson (1984) for hazelnut (*Corylus avellana*) micropropagation. This author related the induction of multiple shoots when he used a combination of 8.87 μM BAP and 4.92 to 9.84 μM 2-iP. For papaya, Kataoka and Inoue (1992) also observed that the mean number of newly formed shoots was higher in the presence of both cytokinins than with BAP alone. Furthermore, symptoms of hyperhydricity appeared when BAP concentration was superior to 2.5 μM . In apple scion cultivars 'Jonagold' and 'Royal Gala' as well, a combination of BAP and 2-iP promoted stem elongation and yielded high quality shoots (Sriskandarajah *et al.*, 1990).

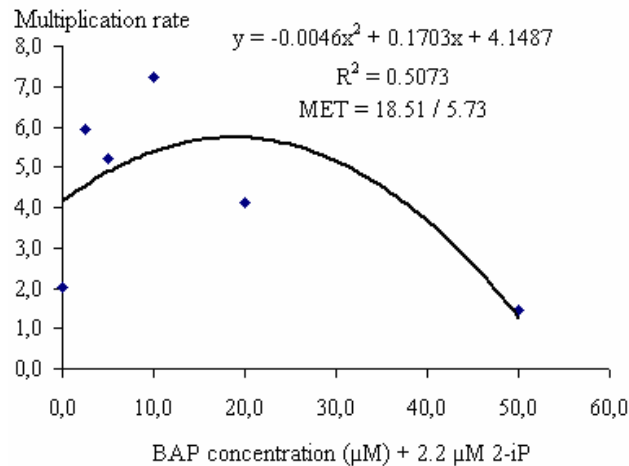


FIGURE 7: Multiplication rate of aerial parts of *Swietenia macrophylla*, 45 days after inoculation in function of concentrations of BAP combined with 2.2 μM 2-iP.

FIGURA 7: Taxa de multiplicação de partes aéreas de *Swietenia macrophylla*, 45 dias após a inoculação, em função de concentrações de BAP combinadas com 2.2 μM de 2-iP.

When QL mineral medium supplemented with BAP and 2-iP was used, multiplication rates were lower than 1.0. The correlation between BAP concentrations and multiplication rate was 0.99, represented by a third degree polynomial equation ($R^2 = 0.99$), as indicated in Figure 8.

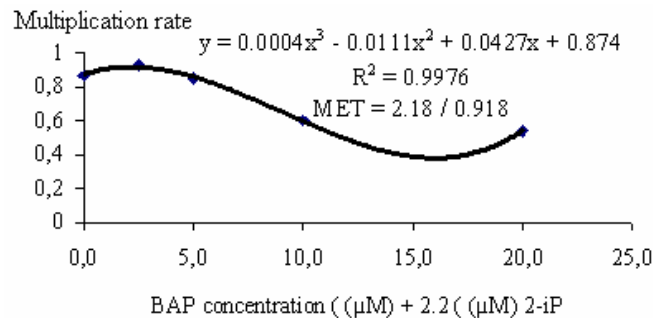


FIGURE 8: Multiplication rate of aerial parts of *Swietenia macrophylla*, 45 days after inoculation, in function of concentrations of BAP combined with 2.2 μM 2-iP.

FIGURA 8: Taxa de multiplicação de partes aéreas de *Swietenia macrophylla*, 45 dias após a inoculação em função de concentrações de BAP (μM) combinadas com 2.2 (μM) de 2-iP.

However, high numbers of buds per shoot were recorded. The correlation between BAP concentrations and bud mean number was 0.99, represented by a third degree polynomial equation ($R^2 = 0.99$) (Figure 9).

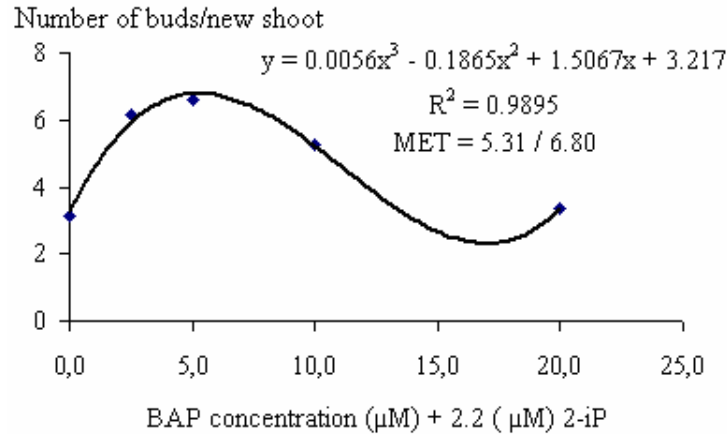


FIGURE 9: Number of buds/new shoot of *Swietenia macrophylla* 45 days after inoculation in function of concentrations of BAP combined with 2.2 µM 2-iP.

FIGURA 9: Número de gemas/broto de *Swietenia macrophylla* 45 dias após a inoculação em função de concentrações de BAP combinadas com 2.2 µM de 2-iP.

The buds remained small and it was not possible to separate them. The mean shoot size for all treatments was as follows: 47.4% of shoots presented a length inferior to 0.5 cm, 47.6% between 0.5 and 1 cm and only 5% of them were superior to 1.1 cm (Figure 10). When culture medium contained 20.0 µM BAP and 2.2 µM 2-iP, the majority of shoots were shorter than 0.5 cm (Figure 10). When BAP concentration of 2.5 or 5.0 µM was combined with 2.2 µM 2-iP, 60% of shoots had a length between 0.5 and 1 cm (Figure 10). This percentage strongly diminished for 20.0 µM BAP and was lower than 20%. Moreover, shoot necrosis was elevated (50%) in this medium (data not shown).

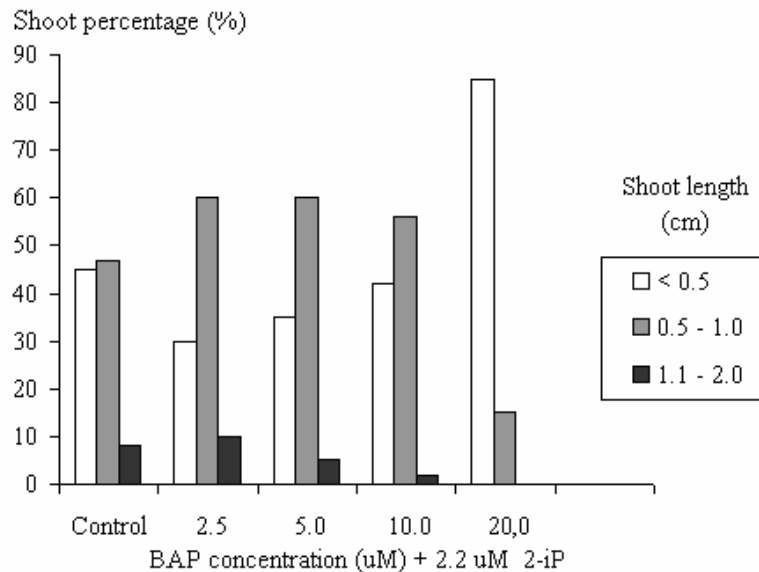


FIGURE 10: Percentage of shoots of *Swietenia macrophylla* King with length < 0.5 cm; between 0.5 and 1.0 cm and between 1.1 and 2.0 cm in function of BAP concentration in QL culture medium containing 2.2 µM 2-iP.

FIGURA 10: Porcentagem média de brotos de *Swietenia macrophylla* com comprimento inferior a 0.5 cm; de 0.5 a 1.0 cm e de 1.1 a 2 cm em função da concentração de BAP no meio de cultura QL contendo 2.2 µM de 2-iP.

When these results were compared to those obtained with the same combinations of BAP and 2-iP on MS culture medium, it looked as if multiplication was reduced on QL culture medium, possibly due to mineral salts composition or inadequate cytokinins concentrations. QL medium has a lower concentration of ammonium, manganese and chlorine ions than MS medium. These differences between results on both media

can also be due to interaction between ion uptake and cytokinins. According to Ramage and Williams (2002), mineral composition may affect the sensitivity of explants to plant growth regulators. There are several studies showing the relationship between both factors. For example, in tobacco leaf discs culture, these authors observed a linear relationship between exogenous BAP supply and the concentration of potassium ions present in the explants. In contrast, Jacoby and Dagan (1970) demonstrated the inhibition of sodium absorption by bean leaves in the presence of BAP.

CONCLUSION

The combination of BAP and 2-iP added to MS culture medium allowed to improve the multiplication of axillary shoots of big-leaf mahogany, which was impossible when these cytokinins were used alone. Such combination needs to be tested during several subcultures. The symptoms of leaf chlorosis were not eliminated, even when QL medium was used, indicating a need for modification of mineral medium.

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