Organogenesis in eggplant (*Solanum melongena* L. cv. Embú) as affected by antibiotics and growth regulators

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Summary: The influence of antibiotics (cefotaxime, timentin, kanamycin and hygromycin) and growth regulators (indolacetic acid and 6-benzylaminopurine) was evaluated on eggplant organogenesis. *Solanum melongena* hypocotyl segments (6 to 10 mm length), taken from 16 to 20-days in vitro grown seedlings, were used as explants. The basic medium was composed by MS salts, Gamborg vitamins and 2% sucrose, solidified with agar 0.8% and pH adjusted to 5.7±0.2. Morphogenesis was impaired at 50 to 100 mg L^{-1} kanamycin and 7.5 mg L^{-1} hygromycin. Both Timentin and cefotaxime reduced the frequency of regenerating explants meanwhile hyperhydricity was not affected. A decrease in root regeneration was observed with increasing cefotaxime concentrations, although, timentin had no effect on root regeneration, as compared to the control treatment. Interestingly, the number of adventitious roots was more noticeable at 0.25 mg L^{-1} IAA plus 0.5 mg L^{-1} BAP. However, if just IAA was added led to higher number of regenerated roots compared to other treatments.

Key words: *Solanum melongena*, antibiotics, morphogenesis, organogenesis, indolacetic acid, benzylaminopurine

Introduction

Solanum melongena L. regeneration is easily attained by means of in vitro somatic embryogenesis and organogenesis. Being an amenable species to in vitro morphogenesis implies in possible uses as a model plant in tissue culture, cellular biology, somaclonal variation, and biotechnology approaches. Transgenic eggplants were obtained mostly by organogenesis (*Alicchio* et al. 1982, *Guri & Sink* 1988, *Filippone & Lurquin* 1989, *Chen* et al. 1995, *Fári* et al. 1995b, *Iannacone* et al. 1995, *Billings* et al. 1997, *Jelenkovic* et al. 1998, *Picoli* 2000, *Picoli* et al. 2002), although somatic embryogenesis may diminish the possibility of chimeras (*Filippone & Lurquin* 1989). *Eady & Lister* (1998) highlighted that the success of plant transformation demands a DNA delivery system, a reliable plant regeneration protocol, and an efficient system for selecting the transgenic individuals.

Research on eggplant in vitro regeneration responses indicates strong evidences of interactions among genotype, explant, genotype-explant, with the regeneration protocols (*Alicchio* et al. 1982, *Gleddie* et al. 1983, *Fobert & Webb* 1988, *Filippone & Lurquin* 1989, *Saito & Nishimura* 1994, *Sharma & Rajam* 1995, *Billings* et al. 1997). Impediment such as somaclonal variation, hyperhydricity and abnormal plant regeneration might also be observed in plant tissue culture (*Fontes* et al. 1999, *Kaeppler* et al. 2000, *Jain* 2001, *Picoli* et al. 2001).

Apart the selection alternatives (*Goldsbrough* et al. 1993, *Hashimoto* et al. 1999, *Negrotto et al.* 2000, *Wang* et al. 2000) and current discussion on the use of antibiotics as selective agents (*Dunwell* 2000, *Otoni* et al. 2002), resistance to such compounds is far the most used methodology. Various selective agents are available, as different plant species react differentially to a particular selective agent (*Eady & Lister* 1998). Selection of eggplant transformed cells is mostly performed with kanamycin, although low efficiencies are reported to some species (*Pollock* et al. 1983, *Mihalka* et al. 1998). Different effects of antibiotics and selective agents on in vitro morphogenesis are also observed elsewere (*Billings* et al. 1997, *Tarré* et al. 1997, *Eady & Lister* 1998). Besides selection, antibiotics such as augmentin, carbenicillin, cefotaxime and timentin are commonly used for suppressing Agrobacterium growth after co-cultivation of plant tissues (*Guri & Sink* 1988, *Billings* et al. 1997, *Nauerby* et al. 1997, Ling et al. 1998, *Costa* et al. 2000). The stimulatory effects of various antibiotics have been observed for a number of species (*Mathias & Boyd* 1986, *Holford & Newbury* 1992, *Yepes & Aldwinckle* 1994, *Sarma* et al. *995, Billings* et al. 1997, *Nauerby* et al. 1997, *Ling* et al. 1998, *Costa* et al. 2000). Among hese antibiotics, timentin has recently been made available with marked efficiency for limination of Agrobacterium, displaying also stimulatory effects on organogenesis (*Nauerby* et al. 1997, *Cheng* et al. 1998, *Ling* et al. 1998, *Costa* et al. 2000, *Hu & Phillips* 2001). To date, effects of timentin as compared to other antibiotics on eggplant organogenesis have not been described yet. Organogenesis responses to growth regulators, indolacetic acid (IAA) and benzylaminopurine (BAP) was approached in previous report (*Picoli* et al. 2001), therefore, the intent of the present work was to evaluate eggplant in

vitro morphogenic responses to antibiotics (timentin, cefotaxime, kanamycin and hygromycin), and rhizogenic responses to growth regulators (IAA and BAP).

Materials and methods

Plant material

Seeds of eggplant (*Solanum melongena* cv. Embú) were purchased from commercial establishments in Viçosa, Brazil. Surface-sterilization was performed by immersion of the seeds in 70% (v:v) ethanol for 1 min, followed by 20 min in a 5% (v:v) sodium hypochlorite solution containing 0.1% (v:v) Tween 20, followed by four rinses in sterile distilled water. Thereafter, seeds were soaked for 24 h, at 26±2 °C, in sterile distilled water on a rotatory shaker (100 rpm). Seeds were germinated in Phytacon® (Sigma Chemical Co., USA) containing 100 ml of a MS-based medium *(Murashige & Skoog* 1962) supplemented with B5 vitamins *(Gamborg* et al. 1968), 100 mg L^{-1} myo-inositol, 2% (w:v) sucrose, pH 5.7±0.2, and solidified with 0.3% (w:v) Phytagel® (Sigma Chemical Co.). Cultures were maintained under 16/8 h light:dark regime, 30 µmol m^{-2} s^{-1} light radiation provided by two fluorescent tubes (Luz do Dia Especial, 20 W, Osram, Brazil). The culture room temperature was kept at 26±2 °C. In vitro-grown seedlings (16–18 days after germination) were used as the source of hypocotyl explants. Hypocotyls were aseptically removed and cut into 6–10 mm length segments. Shoot induction medium consisted of MS basal salts supplemented with B5 vitamins, 100 mg L^{-1} myo-inositol, 2% (w:v) sucrose, and solidified with 0.8% agar (Sigma Chem. Co.) at pH 5.7±0.1, along with IAA (0.0, 0.01, 0.05, 0.1 and 0.25 mg L^{-1}) and BAP (0.0, 0.25 and 0.5 mg L^{-1}). Accordingly, IAA/BAP concentrations 0.0/0.0, 0.0/0.25, 0.0/0.50, 0.01/0.0, 0.01/0.25, 0.01/0.50, 0.05/0.0, 0.05/0.25, 0.05/0.50, 0.1/0.0, 0.1/0.25, 0.1/0.50, 0.25/0.0, 0.25/0.25 and 0.25/0.50, were acknowledged the numbers 1 thru 15.

Likewise, cefotaxime (União Química Farmacêutica Nacional S/A, Brazil) at 0, 250 and 500 mg L^{-1}, and timentin (SmithKline Beecham Farmacêutica, Brazil) at 0, 150 and 300 mg l–1, were evaluated. Regarding the selective antibiotics, kanamycin (0, 50, 100, 150 and 200 mg L^{-1}) or hygromycin (0, 2.5, 5.0, 7.5 and 10.0 mg L^{-1}) (Sigma Chemical Co., EUA) were added to the media, plus 300 mg L^{-1} timentin. The antibiotics were filter-sterilized with Millipore filters (2.5 cm diameter; 0.22 µm; Millex), and added to the medium following autoclaving (1.2 kg cm^{-2} at 121 °C; 15 min) and cooling. After four weeks, data on frequency of explants regenerating shoots (SF), and roots (RF), together with total roots (NR) were evaluated. In the experiments regarding timentin and cefotaxime effects,

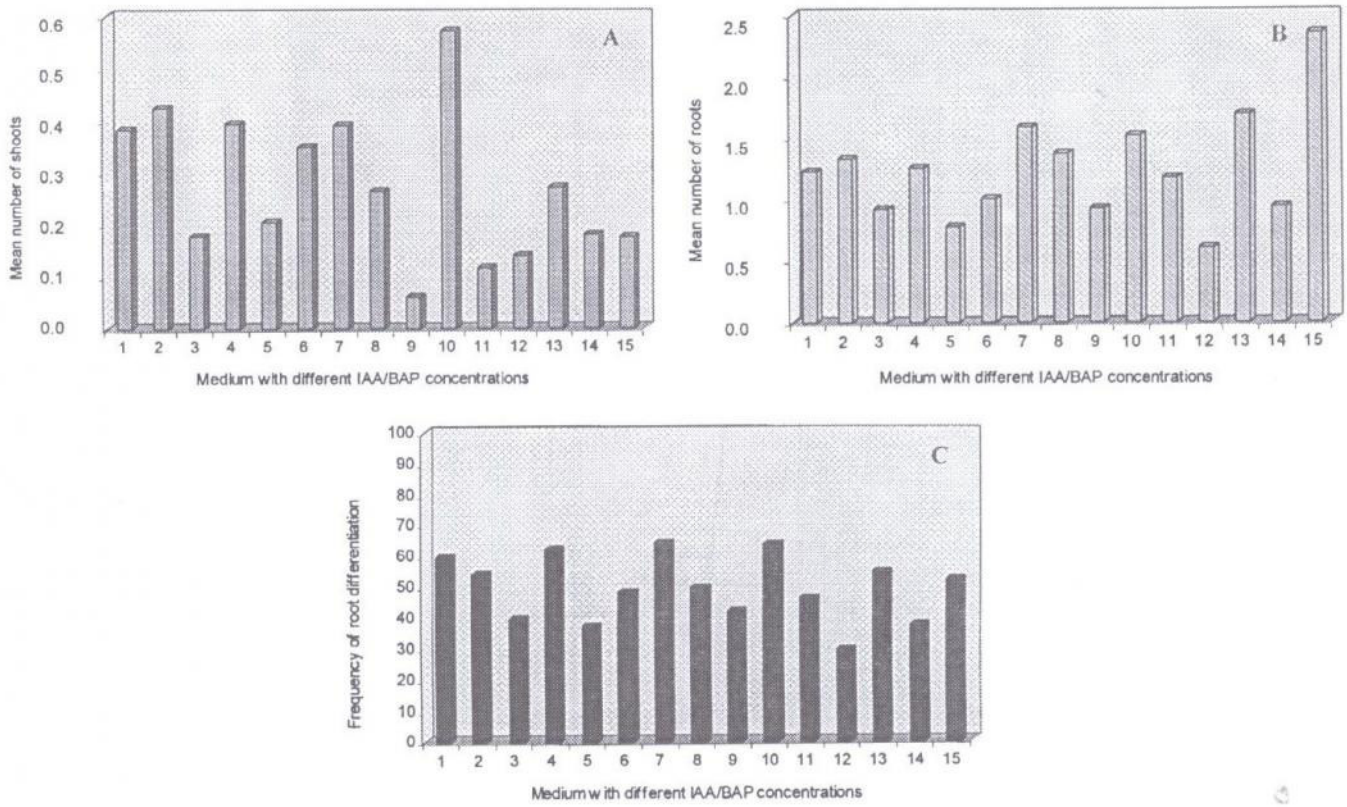

Figure 1 Organogenic responses to different IAA/BAP concentrations. **A** – Mean number of regenerated shoots; **B** – Mean number of regenerated roots. Means are equal to the average of regenerated shoots or roots per explant per plate; and **C** – Root frequency (RF) as influenced by IAA and BAP concentrations. IAA/BAP concentrations (mg L^{-1}) 0.0/0.0, 0.0/0.25, 0.0/0.50, 0.01/0.0, 0.01/0.25, 0.01/0.50, 0.05/0.0, 0.05/0.25, 0.05/0.50, 0.1/0.0, 0.1/0.25, 0.1/0.50, 0.25/0.0, 0.25/0.25 and 0.25/0.50, were numbered 1 thru 15.

hyperhydric shoot frequency (HSF), number of shoots (NS) and number of hyperhydric shoots were also taken into account.

Statistical analysis

Statistical analysis was performed using a completely randomized design. Data were subjected to analysis of variance (ANOVA) and then appropriate regression analysis or Tukey's test (P = 0.05) was used. Treatments had five replicates, with 12 explants per Petri dish. Each experiment was performed twice.

Results and discussion

Effect of growth regulators concentrations

Shoot and root regeneration occurred in all concentrations tested, even in the medium without growth regulators (*Figure 1A, 1B*). Discussion on organogenesis, structural modifications and involvement of BiP (Binding Protein) in hyperhydric shoots were contemplated by *Picoli* et al. (2001). IAA, 0.1 mg L^{-1} and 0.05 mg L^{-1}, were the most the most responsive treatments to root induction frequency (*Figure 1C*). Curiously, the number of adventitious roots was most favored at 0.25 mg L^{-1} IAA plus 0.5 mg L^{-1} BAP. However, treatments that just IAA was added provided higher number of regenerated roots compared to other treatments (*Figure 1B*). Descriptive analysis was performed since none of the tested regression models were adequated to the results.

Sharma and Rajam (1995) obtained a higher number of adventitious shoots of 'Pusa Purple Long' by the use of 11.1 mM BAP plus 2.9 mM IAA. This was tested for 'Embú', although produced inferior results (data not presented). Contrarily to our results, these authors also observed a decrease in the number of shoots with higher IAA concentration. This difference may be due to genotype influence, as previously highlighted (*Alicchio* et al. 1982, *Gleddie* et al. 1983, *Sharma & Rajam* 1995). Similar embryogenesis induction efficiency was established for 'Embú' (*Picoli* et al. 2000) and 'Pusa Purple Long' (*Sharma & Rajam* 1995). Contrasted with the lower organogenesis efficiency, this data suggest that different genes or alleles may be associated to regeneration.

Effects of cefotaxime and timentin concentrations

Lower frequencies of explants regenerating shoots and roots were observed with increasing cefotaxime and timentin concentrations *(Figures 2A and 2B)*. Even though, the

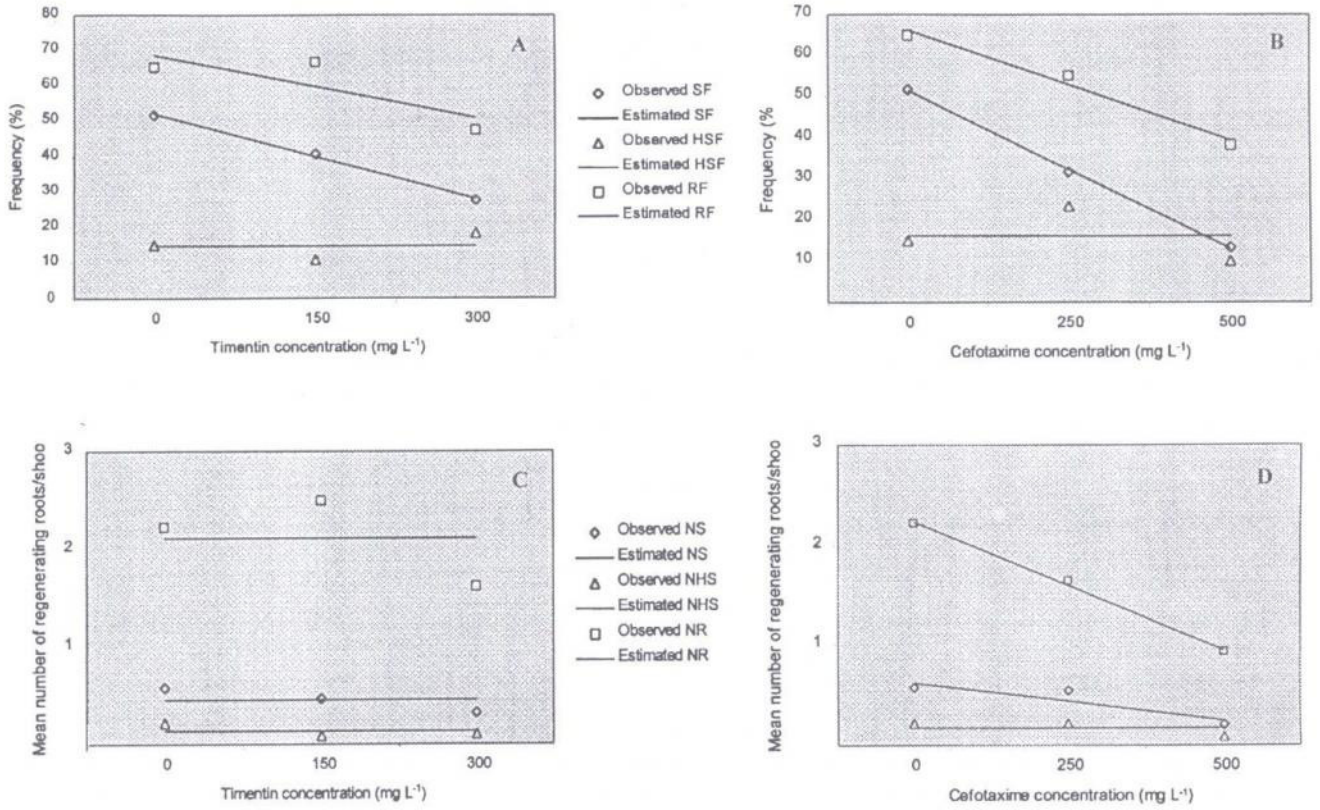

Figure 2 Organogenic responses to different concentrations of timentin and cefotaxime concentrations, media supplemented with 0.1 IAA. **A** – Frequency of explants regeneration shoots in cefotaxime supplemented medium, SF – Shoot frequency; **B** – Frequency of explants regeneration hyperhydric shoots in timentin supplemented medium, SHF – Hyperhydric shoot frequency; **C** – mean number of regenerating shoots and roots in the presence of cefotaxime regeneration shoots; **D** – mean number of regenerating shoots and roots in the presence of timentin, RF – Root frequency. Frequencies are equal to the number of explants regenerating shoots or roots per plate.

Figure 3 Eggplant regeneration within 30 days in presence of inductive and selective medium. **A** – Shoot regeneration in medium supplemented with 0.1 mg L^{-1} IAA and 300 mg L^{-1} timentin; **B** – Explants in medium supplemented with 0.1 mg L^{-1} IAA and 7.5 mg L^{-1} hygromycin.

average number of shoots and roots decreased with cefotaxime (*Figure 2C*), while timentin, in the concentrations tested, had no significant effect over these variables (*Figure 2D*). Both antibiotics did not affected hyperhydricity. Although, considering both antibiotics inhibiting effects on the frequency of explants regenerating shoots (SF), and decreased number of shoots and roots for cefotaxime (*Figures 2A, 2B, 2C and 2D*), they might have caused some stress, as this phenotype was connected to a heat-shock protein also induced by stresses (*Picoli* et al. 2001). Similarly, cefotaxime had deleterious effects over eggplant embryogenesis (*Picoli* et al. 2000). Control plates, containing medium supplemented only with 0.1 mg L^{-1} IAA, naturally displayed shoot development, rhizogenesis and callogenesis (*Figure 3A*).

Shoot regeneration was also decreased in tomato (*Ling* et al. 1998), and inhibited rooting and shoot production in tobacco (*Nauerby* et al. 1997) similarly to our results. Genotype responses interaction with antibiotics is observed since better growth of N. plumbaginifolia cell colonies by use of relate cephalosporins and penicillins were obtained (*Pollock* et al. 1983). *d'Ultra Vaz* et al. (1993) reported that cefotaxime additioned to the medium was essential for passion fruit (*Passiflora edulis f. flavicarpa*) cell division. Cefotaxime positive effects on apple (*Yepes & Aldwinkle* 1994) and Pelargonium x domesticum (*Barret & Cassells* 1994) regeneration, negative on Antirrhinum majus (*Holford & Newbury* 1992) and Antirrhinum majus, Arabidopsis, Solanum tuberosum (*Nauerby* et al. 1997), and, no effects on apple (*Hammerschlag* et al. 1997), reinforce the interaction hypothesis.

Ticarcillin plus potassium clavulanate stimulated callus and shoot formation in L. esculentum Mill. var. Moneymaker (*Ling* et al. 1998). Costa (1998) observed analogous results with 'IPA-5' e 'IPA-6', with 300 mg L^{-1} timentin supplemented medium. *Costa* et al. (2000) detected that timentin added to four different regeneration tomato medium led to increased shoot regeneration and number of roots. Timentin positive effect on soot regeneration of 'Petit Havana' tobacco was also verified (*Nauerby* et al. 1997). *Billings* et al. (1997) reported that 300 mg L^{-1} augmentin induced more shoots in eggplant leaf explants as compared to carbenicillin and cefotaxime. Similarly, *Picoli* et al. (2000) observed that timentin did not affect embryo number and increased callus weight while cefotaxime increased callus weight, although drastically reduced embryo development.

Regeneration response, Agrobacterium-overgrowth control efficiency were improved by using a combination of 250 mg L^{-1} ticarcillin which stimulated regeneration, and 250 mg L^{-1} which controlled Agrobacterium overgrowth (*Hu & Phillips* 2001). *Holford & Newbury* (1992) demonstrated that antibiotics regeneration stimuli might be related to penicillin metabolism. One of the products is the phenyl acetic acid, a natural weak auxin that might be contributing to morphogenic responses. Again, the variability in the responses to antibiotics and its concentrations among species and cultivars takes us to the interaction hypothesis, which makes issues on plant-antibiotic interaction a prerequisite in tissue culture studies.

Effects of kanamycin and hygromycin concentrations

Kanamycin (100 mg L^{-1}) completely inhibited organogenesis. A decrease in morphogenesis was observed with increasing hygromycin concentrations, and was completely inhibited at 7.5 mg L^{-1} (*Figure 3B*). Although, some root apices were observed in 7.5 mg L^{-1} and 10 mg L^{-1} hygromycin, soon they turned brown and died. *Picoli & Otoni* 2002) reported that one day in somatic embryogenesis-induction medium was enough for inducing somatic embryogenesis, callogenesis and rhizogenesis, even when the explants were transferred to a medium without growth regulators. It was highlighted the differentiation of somatic embryos in selective medium containing 5 mg L^{-1} NAA, although embryo had no further development in medium supplemented with 10 mg L^{-1} hygromycin. *Picoli* et al. (2000) did also observed that increasing hygromycin concentrations restrained eggplant somatic embryo development albeit the presence of globular embryos, without additional progress, up to 15 mg L^{-1} hygromycin. Such data is very important since in transformation protocols explants

are maintained in induction medium for 24–72 hours in the absence of selective antibiotics, in conditions where cells are already engaged on morphogenic response processes.

Kanamycin and hygromycin efficiently suppressed morphogenesis, even considering lower hygromycin concentrations (*Figures 4A, 4B, 4C and 4D*). Aminoglicosilates antibiotics such as kanamycin, act inhibiting protein synthesis in mitochondria and chloroplast, resulting in chlorosis and plant tissue growth inhibition (*Weide* et al. 1989; *Eady & Lister* 1998). Meanwhile, hygromycin inhibit protein synthesis in eucariont systems (*Eady & Lister* 1998).

In most reports, kanamycin was used for transgenic eggplant cell selection (*Alicchio* et al. 1982, *Gleddie* et al. 1983, *Guri & Sink* 1988, *Fobert & Webb* 1988, *Filippone & Lurquin* 1989, *Rotino & Gleddie* 1990, *Saito & Nishimura* 1994, *Sharma & Rajam* 1995, *Billings* et al. 1997). Eggplant 'Hibush' was sensitive to a range of 10 to 100 µg ml^{-1} kanamycin, where no growth was observed (*Billings* et al. 1997). The authors obtained a higher proportion of GUS positive plants at 50 µg ml^{-1} kanamycin, while no regenerants were observed at concentrations higher than 70 µg ml^{-1}. Some researchers support that the 0use of higher antibiotics doses is not a good strategy for selection of transformed cells, once that selection pressure may inhibit even differentiation of transgene inserted cells (*Peña* et al. 1995a, 1995b). Although, it must be taken into account, that a lower selection may facilitate regeneration of chimeras.

Pollock et al. (1983) reported different toxicity levels of aminoglicosides in *N. plumbaginifolia* cells. The pH dependent activity of these antibiotics is a disadvantage to their use in plant tissue culture. Even though, kanamycin use is almost unanimity for eggplant transformed cell selection (*Guri & Sink* 1988, *Filippone & Lurquin* 1989, *Rotino & Gleddie* 1990, *Fári* et al. 1995b, *Chen et al.* 1995, *Billings* et al. 1997, *Szász* et al. 1998), except for *Guri & Sink* (1988), which did not obtained resistant plants, and *Picoli* (2000), that recovered somatic embryos resistant to hygromycin.

Torregrosa & Bouquet (1997) employed kanamycin and hygromycin for selecting Vitis transformed cells. Antibiotic tolerance was very low in the clones transformed with A. rhizogenes, where growth was inhibited in 1 mg L^{-1} hygromycin. Although, hygromycin caused root apices necrosis and kanamycin did not, morphogenic responses varied among the clones. *Péros* et al. (1998) verified that the adequate concentration of kanamycin and hygromycin for selection of transformed Vitis cells was 4 mg L^{-1} and 0.8 mg L^{-1}, respectively. Transformed eggplant somatic embryos selection was also more efficient when hygromycin was used, although organogenesis derived shoots could not be recovered. (*Picoli* 2000).

The use of antibiotics as geneticin, hygromycin, methotrexate and phosphonitricin exhibited better results, as selective agents impairing C. annuum organogenesis, as

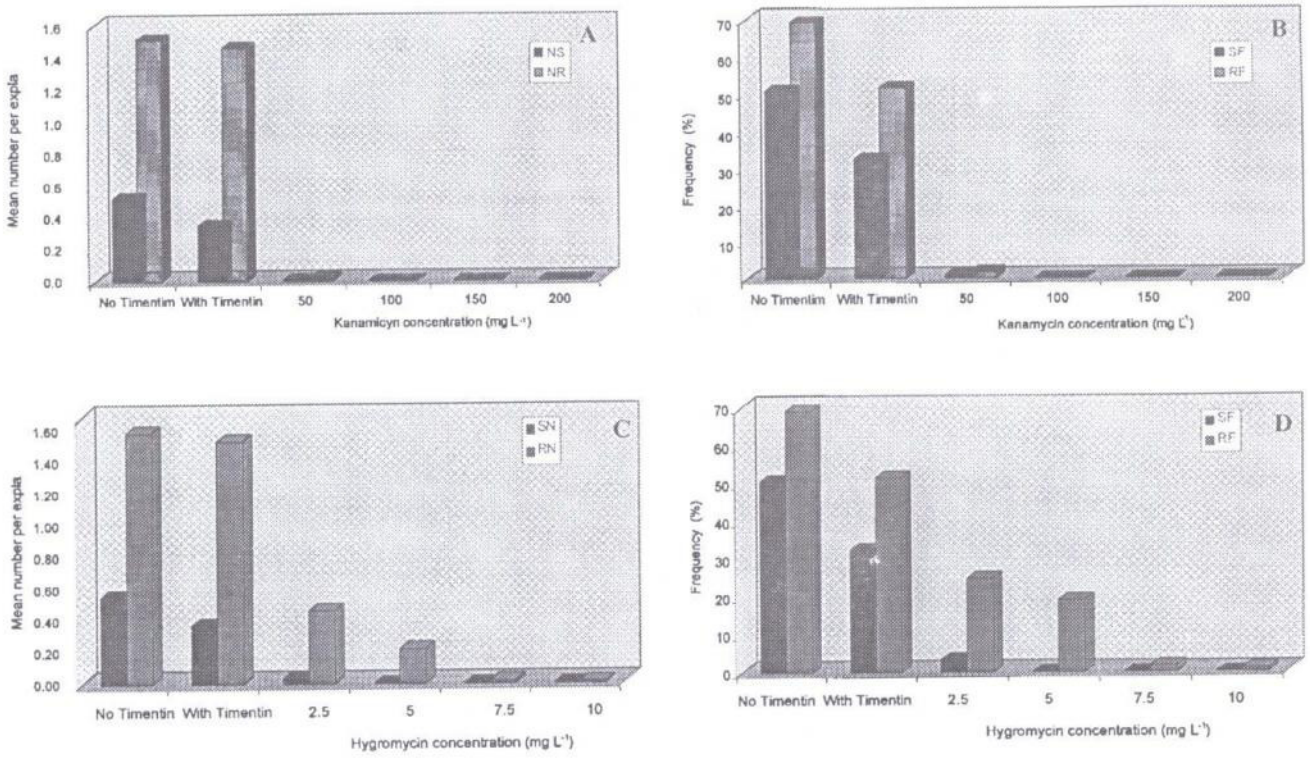

Figure 4 Effects of kanamycin hygromycin supplemented medium to organogenesis of eggplant hypocotyl explants. **A** – Frequency of explants regeneration shoots and roots in medium supplemented with kanamycin; **B** – Number of explants regeneration shoots and roots in medium supplemented with kanamycin; **C** – Frequency of explants regeneration shoots and roots in medium supplemented with hygromycin; and **D** – Number of shoots and roots regenerated in medium supplemented with kanamycin. NS – Number of shoots; NR – Number of roots. SF – Shoot frequency; RF – Root frequency. Frequencies equal to the number of explants regenerating shoots or roots per plate. NS and NR are equal to mean number of shoots or roots regenerating per explant per plate, respectively.

compared to kanamycin (*Mihalka* et al. 1998). The same was observed for eggplant, where lower hygromycin concentrations could restrict morphogenesis as compared to kanamycin.

It is explicit the importance of the use of selective markers, and antibiotics used for eliminating Agrobacterium, in transformation protocols, and the need for adjustments for each species and maybe for the genotype in study. Accordingly to the literature and data for available for other species, hygromycin was also more effective in restraining organogenesis in eggplant. Similarly, timentin is a good option for transformation protocols since did not caused prejudice to the number of organogenic events. This data will support further studies on eggplant genetic transformation that are being held in our laboratory.

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