Kanamycin Sensitivity of Mango Somatic Embryos

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Abstract. Mango (*Mangifera indica* L.) somatic embryos representing various developmental stages were subjected to various concentrations of kanamycin in the culture medium. The level of kanamycin necessary for growth inhibition was dependent on the size and stage of the somatic embryos at the time of treatment and the kind of exposure. Growth of proembryos in liquid suspension was arrested at 12.5 $\mu\text{g}\cdot\text{ml}^{-1}$, while the maturation of later stages of somatic embryos on solid medium was inhibited at 200 $\mu\text{g}\cdot\text{ml}^{-1}$.

Genetic transformation is a potential tool for the improvement of crop plants. Recombinant DNA techniques for introducing foreign genes into plants involve the use of dominant selectable markers that are expressed at the cell and whole-plant levels. The most widely used and tested marker is the gene for neomycin phosphotransferase (NPT II) (EC 2.7.1.95), which confers resistance to the antibiotic kanamycin (Bhatia et al., 1986; Fraley et al., 1986; Schell, 1987).

Mango is a tropical fruit of major economic importance. Somatic embryogenesis from nucellar explants has been demonstrated in both monoembryonic and polyembryonic mango cultivars (Litz, 1984; Litz et al., 1982). As a preliminary step in genetic transformation experiments involving the kanamycin resistance marker gene, we have carried out a detailed investigation to determine the toxic level of the antibiotic on mango somatic embryos at various stages of development. McGranahan et al. (1988) previously demonstrated that walnut embryogenic proembryos can be used as explants for Agrobacterium-mediated transformation.

Embryogenic cultures were initiated from mango nucellus according to procedures described elsewhere (Litz, 1984; Litz et al., 1982, 1984). Suspension cultures of proembryonic masses and cell aggregates were grown in maintenance medium (DeWald et al., 1989a) consisting of B-5 major salts (Gamborg et al., 1968), MS minor salts and organics (Murashige and Skoog, 1962), 6% (w/v) sucrose, 400 mg glutamine/liter, and 4.5 μM 2,4-D.

Growth responses of mango somatic embryos to kanamycin sulfate (Sigma, St. Louis) were tested at four developmental stages. Most experiments were carried out using 'Parris', a polyembryonic cultivar, or 'Tommy Atkins', a monoembryonic cultivar. Initial ex-

periments indicated that small proembryos were more sensitive to kanamycin than those of later stages of embryogeny. Therefore, the concentration range of kanamycin selected for each experiment was dependent on the size and stage of the somatic embryos at the time of exposure to the antibiotic. Increase in fresh weight was calculated as the ratio of final to initial weight.

Stage 1. Proembryos of 'Parris' at the 8-40 16-cell stage of development were obtained by passing embryogenic suspensions through 300- μm mesh nylon filtration fabric. The fine fraction containing embryos of size <300 μm was retrieved, and ≈ 16 mg of proembryos was inoculated into 50 ml of liquid selection medium in 125-ml Erlenmeyer flasks. Selection medium consisted of the maintenance formulation with filter-sterilized kanamycin sulfate at 0, 12.5, 25, or 50 $\mu\text{g}\cdot\text{ml}^{-1}$. Each treatment consisted of five replicates.

Stage 2. Larger proembryos of 'Parris' than used in stage 1 were obtained from suspensions that had been subculture from maintenance medium into liquid embryogenesis medium, i.e., maintenance medium without 2,4-D (DeWald et al., 1989a), and passed through 1000- μm mesh nylon filtration fabric. About 58 mg of the larger proembryos, but <1000 μm across, were inoculated into 50 ml of liquid selection medium in 125-ml Erlenmeyer flasks. Selection medium consisted of the maintenance formulation with filter-sterilized kanamycin sulfate at 0, 25,

50, and 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Each treatment consisted of five replicates.

Stage 3. Globular somatic embryos of 'Parris' and 'Tommy Atkins' were collected after passage of the suspensions through 2500- μm mesh nylon filtration fabric. A packed volume of 3 ml of somatic embryos, obtained by centrifugation of the fine fraction (embryo size < 2500 μm) at 100 $\times$ g for 5 min, was plated on solid medium containing B-5 salts, MS organics, 4% (w/v) sucrose and 20% (v/v) filter-sterilized coconut water with filter-sterilized kanamycin sulfate at 0, 50, 100, or 200 $\mu\text{g}\cdot\text{ml}^{-1}$. Each treatment consisted of five replicates per cultivar.

Stage 4. Somatic embryos at the torpedo (20 to 30 -mm long) stage of development were obtained following the procedure of DeWald et al. (1989b). These were subculture from embryogenesis medium onto solid maturation medium, i.e., maintenance medium without 2,4-D and with 4% (w/v) sucrose, and 20% (v/v) filter-sterilized coconut water. The effect of kanamycin on subsequent development of torpedo-stage mango somatic embryos was determined on medium with filter-sterilized kanamycin sulfate at 0, 20, 50, 100, or 200 $\mu\text{g}\cdot\text{ml}^{-1}$. The number of embryos that germinated or formed secondary embryos was scored after 45 days in culture. The experiment was replicated with 10 petri plates per treatment with an average of 12 embryos per plate.

The pH of the media was adjusted to 5.8 before autoclaving at 121C and 1.1 kg $\cdot\text{cm}^{-2}$ for 20 min. Solid medium was gelled with 0.17% Gelrite. All cultures of proembryos and globular embryos were kept in darkness at 25 $\pm$ 2C, whereas germinating somatic embryos were exposed to a 16-hr photoperiod with a light intensity of 25 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Suspension cultures were maintained on a gyratory shaker at 120 rpm and were subculture at 5-day intervals.

The normal (control) increase in fresh weight of proliferating cultures of stage 1 proembryos in maintenance medium was at a ratio of 466:1, whereas complete growth inhibition was observed at 12.5 $\mu\text{g}\cdot\text{ml}^{-1}$ or higher concentrations of kanamycin during a culture period of 30 days. Kanamycin at 5 $\mu\text{g}\cdot\text{ml}^{-1}$ did not cause any noticeable reduction in growth rate (data not shown). In embryogenesis medium, development of Stage 2 proembryos into early heart-shaped embryos was initiated after $\approx$ 42 days (control),

Table 1. Effect of kanamycin on germination and secondary somatic mango embryo formation at Stage 4.^{1,2}

Kanamycin concn ($\mu\text{g}\cdot\text{ml}^{-1}$)	Germinating somatic embryos (%)	Embryos forming secondary somatic embryos (%)	Average fresh wt of embryos $\pm$ SE (g)
0	39.58	20.83	0.983 $\pm$ 0.101
20	40.00	17.5	0.861 $\pm$ 0.095
50	20.45	4.55	0.537 $\pm$ 0.088
100	13.33	0.0	0.406 $\pm$ 0.059
200	3.64	0.0	0.204 $\pm$ 0.052

¹Culture period 65 days.

²Average number of embryos scored per treatment: 120.

while in kanamycin-containing medium, further development of proembryos was inhibited. These cultures became brown. For these, the final to initial weight ratio was 250 for the control and ≈ 1 for those treated. Kanamycin tolerance was similar for 'Tommy Atkins' and 'Parris' when measured at the most advanced stage of proembryo development (2500 μm). Somatic embryo development was completely inhibited by kanamycin at 200 $\mu\text{g}\cdot\text{ml}^{-1}$ in both 'Tommy Atkins' and 'Parris'. In selection media with 50 or 100 μg kanamycin/ml only one to two somatic embryos developed normally on each petri plate, whereas an average of more than 100 heart-shaped somatic embryos developed on each petri plate in the control. Germination of mature somatic embryos and the proliferation of secondary embryos from their hypocotyls were also reduced in 'kanamycin-containing medium' (Table 1). When germinating somatic embryos were transferred to fresh medium with the same concentration of kanamycin and exposed to light, control cultures without kanamycin produced lateral roots and green cotyledons, whereas the media with kanamycin (50 $\mu\text{g}\cdot\text{ml}^{-1}$ and higher) inhibited hypocotyl elongation and radicle emergence. Chlorophyll impairment in the cotyledons was observed only at 100 and 200 μg kanamycin/ml, but not at the lower concentrations. At 200 $\mu\text{g}\cdot\text{ml}^{-1}$ the somatic embryos became black and necrotic. The average fresh weight of mature somatic embryos was also reduced when they were subculture on kanamycin-containing media.

The sensitivity of mango somatic embryos

to kanamycin varied with respect to the stage of development of the somatic embryos at time of exposure to the antibiotic and to the nature of kanamycin exposure. Early Stage 1 proembryos consist of very few cells that are fully bathed in liquid selection medium. This should prevent the occurrence of "escape cells" in screening experiments. Although 12.5 μg kanamycin/ml drastically affected growth of the smaller (300- μm size) proembryos, embryo maturation and germination still occurred with kanamycin at 20 $\mu\text{g}\cdot\text{ml}^{-1}$ (Table 1). Kanamycin at 100 to 200 $\mu\text{g}\cdot\text{ml}^{-1}$ had a negative effect on maturation of somatic embryos. The toxic level of the antibiotic can vary not only from plant to plant but also in various explants of the same species (Guerche et al., 1987; Mathews, 1988). An understanding of kanamycin toxicity at various stages of development is important in any screening procedure. In transformation experiments involving the kanamycin resistance marker gene, transformed Stage 2 mango somatic embryos (< 1000- μm size) might be selected at a relatively low kanamycin concentration (≤ 25 $\mu\text{g}\cdot\text{ml}^{-1}$) in suspension cultures. Subsequent confirmation of tolerance during the maturation stage of development would require a higher kanamycin concentration.

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