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In Vitro Culture of Rudimentary Embryos of *Ilex paraguariensis:* Responses to Exogenous Cytokinins

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Abstract. The effects of benzyladenine (BAP), kinetin (KIN), zeatin (ZEA), isopentenyladenine (2iP), and thidiazuron (TDZ) were studied on in vitro growth of rudimentary embryos of *Ilex paraguariensis* St. Hil. Heart stage zygotic embryos were removed from seeds of immature, light green fruits and cultured aseptically on quarter-strength Murashige and Skoog medium containing 3% sucrose, 0.65% agar, and supplemented with or without three concentrations of BAP, KIN, ZEA, 2iP, or TDZ. Cultures were incubated in darkness at 27 ± 2°C. Media containing 4.4×10^{-6} M BAP, 4.6×10^{-6} M KIN, or 4.9×10^{-6} M 2iP were totally ineffective in inducing embryo growth after culture for 28 days. However, lower concentrations of these compounds (4.4×10^{-8} M BAP, 4.6×10^{-8} M KIN, 4.5×10^{-8} M ZEA, or 4.9×10^{-8} M 2iP) promoted embryo growth. TDZ at 9.9×10^{-9} M, 9.9×10^{-8} M, or 9.9×10^{-7} M induced embryo growth at similar rates. The maximum percentage of embryos converted to seedlings was achieved when the medium was supplemented with 4.5×10^{-7} M ZEA.

Key Words. *Ilex paraguariensis*—Aquifoliaceae—Cytokinin—In vitro culture—Embryo—Seed germination

The genus *Ilex* L. (Aquifoliaceae) consists of many species, scattered widely throughout the world, occurring in temperate and tropical regions of both hemispheres (Hu 1989). The taxonomic description of all taxa of *Ilex* is incomplete, and it is estimated that the genus consists of more than 300 species (Giberti 1995, Hu 1989). In some regions of Argentina, Brazil, and Paraguay, *Ilex paraguariensis*, occurring as a tree or a shrub, is economically the most important species of the genus because of the value of its leaves for making a stimulatory beverage named *maté.* Like other species of *Ilex* (Hu 1975, Ives 1923), the seeds of *I. paraguariensis* contain rudimentary embryos that remain in the immature, heart-shaped stage a long time after fruits reach maturity. Niklas (1987) reported that when fruits of *I. paraguariensis* are ripe only about 1% of the seeds contain mature embryos, and 99% of the seeds have embryos either in the heart stage (70%) or torpedo stage (29%). As a result, seed germination is delayed, and a minimum of 5–9 months under appropriate environmental conditions is required for embryo maturation (Fontana et al. 1990).

Acceleration of the maturation of rudimentary embryos at the heart-shaped stage in mature fruits can occur by embryo culture techniques that stimulate rapid embryonic development and high germination rates in various *Ilex* spp. (Hu 1975, 1976, 1989). Embryo culture is very simple to use. In general, the greater the degree of immaturity, the more complex the medium required. Generally, exogenously supplied growth regulators are not required for embryo maturation (Monnier 1995).

For species of *Ilex*, an agar-solidified Linsmaier and Skoog (1965) medium containing sucrose and lacking growth regulators has been employed for culturing rudimentary embryos of 11 species (Hu 1975, 1989). Employing the same nutrient medium with the addition of casein hydrolysate, Ferreira et al. (1991) defined some in vitro conditions for development of excised rudimentary embryos of *I. paraguariensis.* Therefore, the following investigation was undertaken to study the morphogenic responses of in vitro cultured embryos of *I. paraguariensis* as influenced by the addition to or deletion from the culture medium of five different cytokinins, each at three concentrations.

Abbreviations: BAP, benzyladenine; KIN, kinetin; 2iP, isopentenyladenine; ZEA, zeatin; TDZ, thidiazuron.

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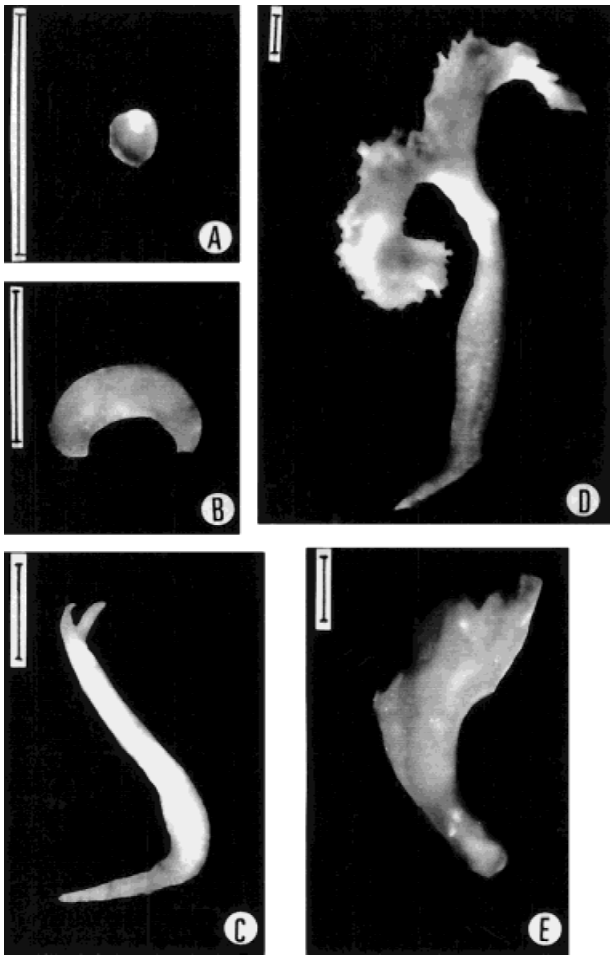

Fig. 1. *Panels A–D,* stages in embryo development of *I. paraguariensis. Bars* represent 1 mm. *A,* excised heart-shaped stage; *B,* torpedo stage; *C,* mature embryo; *D,* seedling; *E,* embryo cultured on 9.9×10^{-7} M TDZ after incubation for 28 days. Note abnormally poor leaf development and suppression of root growth.

Materials and Methods

Immature, light green fruits (drupes) from greenhouse-cultivated plants of *I. paraguariensis* St. Hil. cv. Garruchos 9, provided by Establecimiento Las Marías, Virasoro, Corrientes, Argentina, were harvested December 1995 through January 1996. Fruits were surface sterilized in 70% ethanol for 5 min followed by immersion for 30 min in 1.8% sodium hypochlorite, with two drops of TRITON®. Fruits were then rinsed three times with autoclaved distilled water and kept in sterile water until excision of rudimentary embryos under a stereomicroscope in a laminar flow hood.

Embryos at 0.25–0.26 mm in length at the heart-shaped stage (Fig. 1*A*) were cultured on 3 mL of culture medium in 11-mL glass tubes (one embryo/tube). The tubes were sealed with Resinite AF 5O® (Casco S.A.I.C. Company) and incubated in darkness at 27 ± 2°C. Each treatment was replicated nine or ten times, and each experiment was conducted three times. Data regarding changes in stages in embryo development were recorded weekly for 28 days.

The culture medium was that reported by Rey et al. (1991) for *I. paraguariensis.* It consisted of quarter-strength Murashige and Skoog (1962) medium containing 3% sucrose, 0.65% agar, and supplemented with or without BAP (4.4×10^{-8} M, 4.4×10^{-7} M, or 4.4×10^{-6} M), KIN (4.6×10^{-8} M, 4.6×10^{-7} M, or 4.6×10^{-6} M), 2iP (4.9×10^{-8} M, 4.9×10^{-7} M, or 4.9×10^{-6} M), ZEA (4.5×10^{-8} M, 4.5×10^{-7} M, or 4.5×10^{-6} M), or TDZ (9.9×10^{-9} M, 9.9×10^{-8} M, or 9.9×10^{-7} M). The pH of each medium was adjusted to 5.8 with KOH or HCl before the addition of agar. Tubes were covered with aluminum foil and autoclaved at 1.46 kg/cm^2 for 20 min.

Seedlings bearing two true leaves were transplanted successfully out of aseptic tubes to pots (5-cm diameter) containing vermiculite and were maintained in a growth room at 27 ± 2°C, with a 14-h photoperiod (116 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Relative humidity was maintained at 95–100% during the first 7 days and then decreased gradually until established in a greenhouse.

Results and Discussion

The following sequential stages were observed during in vitro development of excised rudimentary embryos of *I. paraguariensis:* heart-shaped stage (Fig. 1*A*), torpedo stage (Fig. 1*B*), mature stage (Fig. 1*C*), and germination (or seedlings) (Fig. 1*D*). This sequence was described for others species of *Ilex* (Hu 1975, 1976; Niklas 1987). In this study, the earliest visible response (within 7 days of culture) was the gradual change in the shape of the cultured embryos. In most media, the initially heart-shaped embryos were converted into torpedo-shaped embryos. After 14–21 days, mature embryos were observed, whereas the first seedlings were germinated from the embryos after 14–28 days (depending upon the culture medium). The embryos were yellowish white. In vitro germination of the embryos was characterized by elongation of the radicle followed immediately by elongation of the hypocotyl and expansion of the cotyledons. These results agree with those obtained for most species of *Ilex* (Hu 1989), where embryos require about 10–30 days of incubation to complete development and germinate. Ferreira et al. (1991) found that embryos of *I. paraguariensis* cultured at the heart stage completed embryonic development in 15 days, reaching a length similar to that of mature, seed-developed embryos. In contrast, embryos of *Ilex aquifolium* required an incubation period of 6–8 weeks (Hu 1975).

The morphogenic responses of rudimentary embryos under the influence of three concentrations of five cytokinins are presented in Fig. 2. Because 20% of the cultured heart-shaped embryos of *I. paraguariensis* were converted into seedlings when incubated in a medium lacking cytokinin (Fig. 2*A*), this strongly suggests that exogenous cytokinin is not required for embryo maturation. Similar results were reported in other species of *Ilex* (Hu 1989) as well as in many cases, for other plant species (Monnier 1995). However, results presented in Fig. 2 show that the addition of cytokinins to the media could inhibit or modify the rate of embryo development. Media containing 4.4×10^{-6} M BAP, 4.6×10^{-6} M KIN, or 4.9×10^{-6} M 2iP were totally ineffective and were in

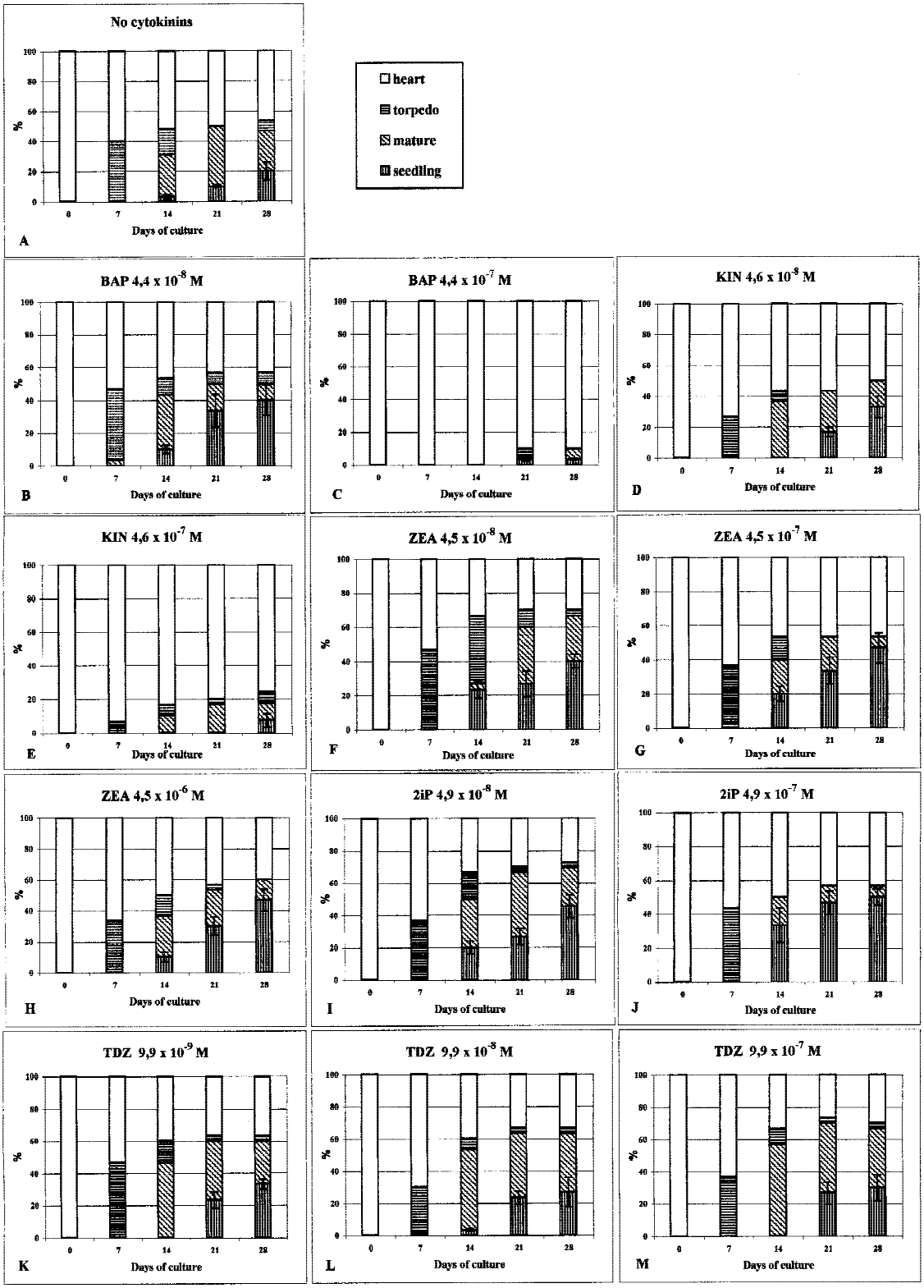

Fig. 2. Effects of cytokinins on in vitro development of rudimentary embryos of *I. paraguariensis.* The figure shows the percentage of heart-shaped embryos converted to torpedo stage, mature stage, and seedling stage at various time points. *Vertical bars* indicate ±S.E.

fact, countereffective for inducing embryo growth (data not presented). After 28 days of culture, 100% of the cultured heart-shaped embryos remained unchanged or exhibited no development. Inhibition caused by cytokinins has been reported for embryos of orchids (Kano 1965, Raghavan and Torrey 1964a). Media containing 4.4×10^{-7} M BAP or 4.6×10^{-7} M KIN also caused a reduction in the percentage of embryos that developed into seedlings (Fig. 2, *C* and *E*). However, 4.4×10^{-8} M BAP, 4.6×10^{-8} M KIN, 4.5×10^{-8} M ZEA, or 4.9×10^{-8} M 2iP resulted in an increase (in some cases approximately 100% more embryos converted to seedlings, Fig. 2 *B, D, F,* and *I*). Similar results occurred when media included 4.5×10^{-7} M or 4.5×10^{-6} M ZEA or with the three tested concentrations of TDZ (Fig. 2 *G, H, K, L,* and *M*) as well as with 4.9×10^{-7} M 2iP (Fig. 2*J*). Although several of these cytokinins produced similar results, we recommend use of 4.5×10^{-7} M ZEA because of the vigor of the resulting seedlings. Germination promotion caused by cytokinins has been reported for embryos of *Acer pseudoplatanus* L. (Pinfield and Stobart 1972). In some cases, low concentrations of cytokinin combined with low concentrations of auxin were beneficial for in vitro growth of young embryos (Raghavan and Torrey 1963, Veen 1963).

Results presented herein suggest that there is great potential for using TDZ for in vitro growth of immature embryos. TDZ was found to be a very useful cytokinin for tissue culture of many woody species (Huetteman and Preece 1993). It is a phenylurea derivative that has been used in tissue culture systems to stimulate axillary shoot proliferation as well as to promote callus formation (Huetteman and Preece 1993). Although TDZ has been employed to induce organogenesis or somatic embryogenesis through in vitro culture of both immature (Beattie and Garret 1995, Goffreda et al. 1995, Norgaard and Krogstrup 1991) and mature embryos (Obeidy and Smith 1993), there are no reports in the literature regarding use of TDZ to stimulate in vitro rudimentary embryo development.

On the other hand, in media containing cytokinins (especially TDZ) some of the embryos grew abnormally, with precocious leaf expansion and suppression of root growth (Fig. 1*E*). Similar results were reported for other plant species (Beattie and Garrett 1995, Raghavan and Torrey 1964b). In this work, callus formation was also detected in embryos cultured in media containing the highest levels of TDZ, but further embryo development was not observed.

After approximately 35–40 days of culture, the plantlets were transplanted successfully to soil. A 90–95% survival rate was obtained when plants were planted in the greenhouse. All of them exhibited a normal phenotype with respect to growth habit and leaf shape.

In conclusion, results herein demonstrate that plantlets of *I. paraguariensis* can be achieved readily by in vitro culture of immature heart stage embryos on a semisolid quarter-strength Murashige and Skoog medium containing 3% sucrose and supplemented with 4.5×10^{-7} M ZEA. However, additional studies are needed to increase the frequency of recovering whole plants from excised immature embryos. It is also necessary to improve the excision procedures with minimal damage to the tender and fragile embryos. It would be particularly useful to improve the rapidity of embryo excision (approximately 200–250 excised embryos/individual/day).

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